PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 31 July 2001 (31.07.01)	
International application No. PCT/US00/16250	**Applicant's or agent's file reference** 0974/2E916-WO
International filing date (day/month/year) 12 June 2000 (12.06.00)	**Priority date** (day/month/year) 11 June 1999 (11.06.99)
Applicant THOMSEN, Gerald, H. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

09 January 2001 (09.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Zakaria EL KHODARY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

HR

PATENT COOPERATION TREATY

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From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: RAINA SEMIONOW
DARBY & DARBY P.C.
805 THIRD AVENUE
NEW YORK, NEW YORK 10022-7513

DUE: OCTOBER 16, 2001
Docketed on 8/24/01 by DD for
Docketed without file ☐

ATTY REVIEWED
DATE

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	16 AUG 2001
Applicant's or agent's file reference: 0974/2E916-WO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/16250	12 JUNE 2000	11 JUNE 1999

Applicant: THE RESEARCH FOUNDATION OF THE STATE OF NEW YORK

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer HOPE ROBINSON
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 0974/2E916-WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/16250	International filing date *(day/month/year)* 12 JUNE 2000	Priority date *(day/month/year)* 11 JUNE 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE RESEARCH FOUNDATION OF THE STATE OF NEW YORK		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09 JANUARY 2001	23 JULY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer HOPE ROBINSON
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16250

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-76 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 77-80 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-25 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16250

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 27-35

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 27-35 .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16250

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. **statement**

Novelty (N)	**Claims**	1-26, 36	**YES**
	Claims	NONE	**NO**
Inventive Step (IS)	**Claims**	1-26, 36	**YES**
	Claims	NONE	**NO**
Industrial Applicability (IA)	**Claims**	1-26, 36	**YES**
	Claims	NONE	**NO**

2. **citations and explanations (Rule 70.7)**

Claims 1-26 and 36 meets the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest isolated SMURF protein. Thus, the claimed invention meets the PCT Article requirement.

NEW CITATIONS

NONE

Suppl mental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07H 21/04; C12N 15/00; C07K 14/00. and US Cl.: 530/350, 329, 328, 300, 326, 327; 435/29, 320.1, 325, 375; 536/23.1.

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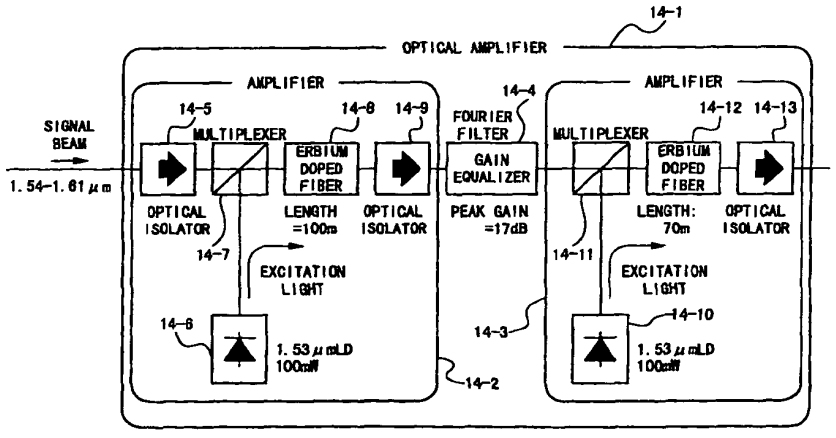
(54) OPTICAL AMPLIFIER AND TRANSMISSION SYSTEM USING THE SAME

(57) An optical amplifier having a two-stage construction using an erbium doped fiber (EDF) as a gain medium. The erbium dopant concentration is 1000 ppm, and the unsaturated absorption coefficient of the signal beam at 1550 nm is 1 dB / m. The length of the EDF 14-8 is 10 m, and the length of the EDF 14 - 12 is 70 m. The excitation light sources 14 - 6 and 14 - 10 are semiconductor lasers of 1.53 μm, and the excitation light power is 100 mW. Multiplexers 14 - 7 and 14 - 11 are inductive multilayer film filters, and the gain equalizer 14 - 4 is a Fourier filter. The peak loss of the Fourier filter is 17 dB. The gain of the EDF 14-8 is 25 dB, and the gain of the EDF 14-12 is 15 dB. Two optical isolators are installed on a pre-stage amplifier, and one on a post-stage amplifier in order to prevent laser oscillation.

FIG.14



EP 0 911 926 A1

Description

Field of th Invention

[0001] The present invention relates to an optical amplifier and a transmission system which uses it, which are necessary in an optical fiber transmission system and optical signal processing system.

Description of Related Art

[0002] The structure of an optical amplifier of the related technology used in an optical fiber transmission system is shown in Figs. 23 ~ 25. Figs. 23, 24, and 25 show respectively the first, second, and third structures of the optical amplifiers of the related technology.

[0003] In Fig. 23, the optical amplifier 23 - 1 comprises an amplifier 23 - 2 and a gain equalizer 23-3. This optical amplifier 23 - 1 is connected to transmission fibers 23 - 4 and 23 - 5. Signal beams with a plurality of wavelengths are incident on this optical amplifier 23-1, and amplified. This amplifier 23 - 2 comprises a gain medium 23 - 6 (a rare-earth element doped fiber or waveguide), an excitation light source 23-7, and an optical part 23 - 8 (multiplexer for excitation light and signal beam, a light isolator, etc.) disposed on the pre-stage of a gain medium 23 - 6, and an optical part 23 - 9 (optical isolator, etc.) disposed on the post-stage of the gain medium 23 - 6 (see Citation Massicott et al., Electron. Lett., vol. 26, No. 20, pp. 1645 - 1646, 1990).

[0004] The gain characteristics of the optical amplifier 23 - 1 whose structure is shown in Fig. 23 are shown in Figs. 26A ~ 26C. Fig. 26A shows the wavelength dependency of the gain of the gain medium 23 - 6. In Fig. 26A, the peak value of the gain is 30 dB, the gain-flattened bandwidth (for example, the 3 dB gain-reduction bandwidth) is 10 nm. The loss of the gain equalizer 23 - 3 is shown in Fig. 26B. The peak value of this loss is about 10 dB. The value obtained by subtracting the loss of Fig. 26B from the gain of Fig. 26A is the gain of the optical amplifier 23 - 1, and this is shown in Fig. 26C. For simplification, the loss of the optical part 23 - 8 and the optical part 23 - 9 are ignored. By using the gain equalizer 23 - 3, the gain-flattened bandwidth is increased by about 30 nm. In this manner, as long as the signal beam wavelength intervals are equal, if the gain-flattened bandwidth is widened, it is an advantage that signal beams of more wavelengths (and therefore more channels) can be amplified with an identical gain.

[0005] Fig. 24 has the same gain characteristics as Fig. 23, but compared to Fig. 23, this structure of an optical amplifier has lower noise. The difference between this figure and Fig. 23 is that in this figure two excitation light sources 23 - 7 and 24 - 3 with different excitation light wavelengths are used. The wavelength of the excitation light which is output by excitation light source 24 - 3 is shorter than the wavelength of the excitation light output by excitation light source 23 - 7, and the upper part of the gain medium 23 - 6 (with respect to the input direction of the signal beam) is excited to a higher population inversion stat in comparison to Fig. 23 (see Citation Massicott et al., Electron. Lett., vol. 28, No. 20, pp. 1924 - 1925, 1992).

[0006] Fig. 25 is an optical amplifier with a structure analogous to the structure of the present invention, although the widening of the bandwidth of the gain was not planned. The amplifier is divided into a pre-stage (amplifier 25 - 2) and a post-stage (amplifier 25 - 3), and a band restricting optical filter or a dispersion compensator is disposed therebetween. The signal beam is generally a single wavelength. When a band limiting optical filter is used, because the gain medium is divided into two stages, degradation of the amplification characteristics due to laser oscillation or amplified spontaneous emission light is not incurred, and a high gain is possible. When using a dispersion compensator, it is possible to eliminate degradation of the signal to noise ratio due to loss in the dispersion compensator (see Citation Masuda et al., Electron. Lett., vol. 26, No. 10, pp. 661 - 662, 1990).

[0007] In the structures shown in Fig. 23 and Fig. 24, flattened-gain dependence of the flat-gain bandwidth and equalizer loss dependency of the optical amplifier saturation power are shown respectively in Fig. 9A and Fig. 9B. In Fig. 9A, the flattened-gain bandwidth decreases along with the increase in the flattened-gain, and the flattened-gain is limited to 30 dB because of amplification characteristics degradation due to laser oscillation and amplified spontaneous emission light. In contrast, in Fig. 9B, the optical amplifier saturation output power remarkably decreases along with the increase in the equalizer loss. However, the drawback occurs that in obtaining a wide flattened-gain bandwidth, it is difficult to obtain a wide flattened-gain bandwidth while maintaining a large optical amplifier saturation output power because of a necessarily large equalizer loss.

[0008] The object of the present invention is to resolve these problems, and provide a wide bandwidth optical amplifier.

Summary of the Invention

[0009] In order to obtain the above-described object, the present invention provides an optical amplifier provided with a split gain medium wherein a long gain medium using a rare-earth doped fiber as the gain medium is partitioned into two or more stages, two or more amplifiers which include excitation light sources which output excitation light such that the effective excitation wavelength of this gain medium is 1.53 μm, and a gain equalizer which is effective for a wide wavelength band of a gain medium disposed between each amplifier. In this manner, compared to the related technologies, the effect is obtained that the gain-flattened band is wide, and it is possible to realize a high saturation out-

put, low noise optical amplifier.

[0010] In addition, the present invention provides a Raman amplifier provided with a high nonlinear fiber or a dispersion compensation fiber as a Raman amplifier medium, and carries out Raman amplification by this Raman amplifier medium, and a rare-earth element doped fiber amplifier which makes a rare-earth doped fiber the amplification medium. In this manner, the gain bandwidth is flattened and it is possible to structure a broadband lumped constant optical amplifier.

[0011] In addition, the present invention provides an optical transmission system with an optical amplifier as a structural component provided with a Raman amplifier which carries out Raman amplification by a dispersion-compensation fiber wherein a parameter which compensates the dispersion of the transmission path is set, and a rare-earth doped fiber amplifier which uses a rare-earth fiber as an amplifier medium. In this manner, when using a dispersion-compensation fiber as Raman amplifier medium, it is possible to realize large capacity wavelength division multiplex optical transmission because it is possible to compensate the dispersion of the transmission path.

Brief Description of the Drawings

[0012]

Fig. 1 is a block diagram showing a first structure of this invention (in the case of a two-stage gain medium).

Fig. 2 is a block diagram of a second structure of this invention.

Fig. 3 is a block diagram of a third structure of this invention.

Fig. 4 is a block diagram of a fourth structure of this invention.

Fig. 5 is a block diagram of a fifth structure of this invention.

Fig. 6 is a block diagram of a sixth structure of this invention.

Figs. 7A and 7B are graphs showing the characteristics of the first structure of this invention.

Fig. 8 is a graph showing characteristics of the first structure of this invention.

Figs. 9A and 9B are graphs showing the characteristics of the first structure of this invention and the related technology.

Fig. 10 is a graph of the characteristics of the third structure of this invention.

Figs. 11A and 11B show graphs of the structure of a typical example of the related technology.

Figs. 12A and 12B are graphs showing the characteristics of the present invention and the typical example of the related technology.

Fig. 13 is a block diagram showing the structure of a typical example of the present invention.

Fig. 14 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the first embodiment of the present invention.

Fig. 15 is a graph showing the gain spectrum of the first embodiment.

Fig. 16 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the second embodiment.

Fig. 17 is a graph showing the noise index spectrum of the first embodiment and the second embodiment.

Fig. 18 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the third embodiment of this invention.

Fig. 19 is a graph showing the gain spectrum of the third embodiment.

Fig. 20 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the fourth embodiment of this invention.

Fig. 21 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the fifth embodiment of this invention.

Fig. 22 is a block diagram showing an example of the structure of a wide bandwidth optical amplifier according to the sixth embodiment of this invention.

Fig. 23 is a block diagram showing an example of the first structure of the optical amplifier of the related technology.

Fig. 24 is a block diagram showing an example of the second structure of the optical amplifier of the related technology.

Fig. 25 is a block diagram showing an example of the third structure of the optical amplifier of the related technology.

Figs. 26 A ~ C are graphs showing the characteristics of the optical amplifier having the structure in Fig. 23.

Fig. 27 is a block diagram showing a first structure of this invention (a three stage gain medium).

Fig. 28 is a graph showing the characteristics of the gain spectrum of the optical amplifier using Raman amplification.

Fig. 29 is a block diagram of a structure of a seventh embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Fig. 30 is a block diagram of a structure of a eighth embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Fig. 31 is a block diagram of a structure of a ninth embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Fig. 32 is a block diagram of a structure of a tenth embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it

according to this invention.

Fig. 33 is a block diagram of a structure of a eleventh embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Fig. 34 is a block diagram of a structure of a twelfth embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Fig. 35 is a block diagram of a structure of a thirteenth embodiment of the optical amplifier in the optical amplifier or an optical transmission system using it according to this invention.

Figs. 36A and 36B are block diagrams showing the first embodiment of an optical transmission system in the optical amplifier and an optical transmission system using it according to the present invention.

Figs. 37A and 37B are block diagrams showing the second embodiment of an optical transmission system in the optical amplifier and an optical transmission system using it according to the present invention.

Preferred Embodiments of the Present Invention

[0013] First, we will explain the summary of the first through sixth structures of the optical amplifier wherein a long length gain medium is partitioned into several stages, and a gain equalizer connects each partitioned gain medium, then we will explain the first through sixths embodiments.

[0014] Next, we will explain the seventh through thirteenth embodiments of an optical amplifier provided with a Raman amplifier wherein a high nonlinear fiber or a dispersion-compensation fiber is used as a Raman amplification medium.

[0015] Finally, we will show two embodiments of an optical transmission system wherein an optical amplifier, which Raman amplifies by a dispersion-compensation fiber, is used as a component element.

Summary

[0016] The first through sixths structures of the present invention are shown in Figs. 1 to 6.

[0017] Fig. 1, which is the first structure, differs markedly from Fig. 23 of the related technology in that the amplifier is divided into two stages: a pre-stage (amplifier 1 - 2) and a post-stage (amplifier 1 - 3). In addition, Fig. 1 differs markedly from Fig. 25 of the related technology in that the optical part disposed between the pre-stage of the amplifier 1 - 2 and the post-stage of the amplifier 1 - 3 is a gain equalizer 1 - 4, and that the input signal beam is a wide band multiple wavelength beam.

[0018] The gain characteristics of this first structure are shown in Figs. 7A 7B, and 7C. Fig. 7A shows the wavelength dependency of the gain of the gain medium. In Fig. 7A, the peak value of the gain is about 40 dB, and in comparison to the related technology, because there is no degradation of the amplifier characteristics due to laser oscillation and amplified spontaneous light emission, a high value can be obtained. The typical value of the gain of the pre-stage amplifier 1 - 2 is 25 dB, and the typical value of the gain of the post-stage amplifier 1 - 3 is 15 dB. In addition, the gain increases at or above a constant value (for example, 10 dB or 20 dB). Fig. 7B shows the loss of the equalizer 1 - 4. The peak value of this loss is about 10 dB and 20 dB.

[0019] The value derived by subtracting the loss in Fig. 7B from the gain in Fig. 7A is the gain of optical amplifier 1 - 1, and this is shown in Fig. 8. For the sake of simplification, the loss of optical parts 1 - 8 and 1 - 10, and optical parts 1 - 12 and 1 - 14 has been ignored. The gain-flattened bandwidth with the 10 dB loss peak value is 30 nm, and the gain-flattened bandwidth with the 20 dB loss peak value is 50 nm. The gain-flattened bandwidth with a 20 dB flattened-gain value in the related technology is 30 nm as shown in Fig.26C, and the gain-flattened bandwidth with a 20 dB flattened-gain value in the present invention is 50 nm as shown in Fig.8. Due to the structure of the present invention, we can understand that the gain-flattened bandwidth has been remarkably widened

[0020] The dependency of the flattened-gain bandwidth upon the flattened-gain in the present invention is shown in Fig. 9A. In comparison to the related technology, we understand that the gain-flattened bandwidth has been remarkably increased. Fig. 9B shows the dependency of the optical amplifier saturation output upon the equalizer loss in the present invention. In the present invention, because there is an optical amplifier (amplifier 1 - 3) following the gain equalizer 1 - 4, we understand that the saturated output of the optical amplifier does not depend very much on the equalizer loss. In comparison with the related technology, we understand that the saturated output of the optical amplifier has remarkably increased. As shown above, in the first structure of the present invention, it is possible to guarantee a wide gain-flattened bandwidth while maintaining the high optical amplifier saturation output as-is.

[0021] In addition, the structure of the case wherein the gain medium has been partitioned into three stages is shown in Fig. 27. The gain equalizers 1 - 4 and 1 - 4' have been disposed between the three-stage gain medium. Because two gain equalizers 1 - 4 and 1 - 4' are being used, the total peak value of the loss of the gain equalizer can be set at about 30 dB. The gain-flattened bandwidth at this time is 60 nm. Because the gain-flattened bandwidth is 50 nm when the gain medium is divided into two stages, it is possible to enlarge the gain-flattened bandwidth about 10 nm by partitioning into three stages. Moreover, in the figure, an example of a gain medium partitioned into three stages is shown, but it is possible to compose the optical amplifier in which the number of partitions is N (N is an inte-

ger equal to or greater than 2), and an N stage amplifier wherein a partitioned gain-medium is used as a structural component, and N - 1 stage gain equalizer is disposed between these amplifiers. Moreover, by increasing the number of partitions N, it is possible to gradually increase the gain-flattened bandwidth of the optical amplifier within the bandwidth range of the gain medium.

[0022] Fig. 2 shows the second structure of the present invention. In the second structure of the present invention, the amplifiers 1 - 2 and 1 - 3 having the structure (first structure) shown in Fig. 1 are respectively replaced with amplifiers having the structure shown in Fig. 2. Compared to Fig. 1, the amplifier further comprises one more excitation light source. In Fig. 2, for the sake of simplifying the figure, only the points of difference with Fig. 1 are shown for the amplifier 2 - 1 which corresponds to the amplifier 1 - 2 in Fig. 1. This point of difference is similar for the amplifier (not shown) corresponding to the amplifier 1 - 3 in Fig. 1. In comparison with Fig. 1, the present construction is a structure of a lower noise optical amplifier. The difference between Fig. 2 and Fig. 1 is that two excitation source lights 1 - 7 and 2 - 2 with different optical excitation wavelengths are used. The wavelength of the excitation light emitted from excitation light source 2 - 2 is shorter than the wavelength of the excitation light emitted from excitation light source 1 - 7, and in comparison with Fig. 1, the upper part of the gain medium 1 - 9 (with respect to the direction of input of the signal beam) is excited to a higher population inversion state.

[0023] Fig. 3 shows the third structure of the present invention. The difference between this figure and Fig. 23 (related technology) is that a transmission fiber 23 - 4 is used as an amplifier medium, and its excitation light source 3 - 3 is newly installed. The transmission fiber 23 - 4 carries out Raman amplification, and its gain has the characteristic of flattening the wavelength dependency of the gain medium such as a rare-earth doped fiber, etc., that is, equalizing the gain depending on wavelength. That is, the wavelength of the excitation light is set in the short-wave part only of the Raman shift amount (about 110 nm for silica fibers) of the wavelength which produces gain equalization. The gain characteristics of this third structure are shown in Fig. 10. The gain-flattened bandwidth of the total gain (gain medium gain - equalizer loss + Raman gain) is wider than the gain-flattened bandwidth of the gain (gain medium gain - equalizer loss) when Raman amplification is carried out.

[0024] Fig. 4 shows the fourth structure of the present invention. The gain and noise characteristics are similar to those of Fig. 2 (the second structure of the present invention), but the component parts are simpler, cheaper, and the construction becomes more stable. In order to guarantee low noise characteristics, a excitation light source 2 - 2 with a short excitation wavelength is used. Using optical part 4 - 2, a laser ring (optical part 1 - 8 ~ gain medium 1 - 9 ~ optical part 1 - 10 ~ optical part 4 - 2 ~ optical part 1 - 8) using gain medium 1 - 9 as a laser oscillation medium is formed. At this time, the optical part 1 - 8 and the optical part 1 - 10 have a multiplexer and demultiplexer respectively for laser oscillation. This laser oscillation light has an operation similar to the excitation light which the excitation light source 1 - 7 outputs in Fig. 2 (the second structure of the present invention), that is, an operation wherein the gain medium is excited to the desired population inversion state.

[0025] Fig. 5 shows the fifth structure of the present invention. The structure is analogous to that of Fig. 4 (the fourth structure of the present invention), but the propagation direction of the laser oscillation beam is reversed. At this time, the optical part 1 - 8 and optical part 1 - 10 have a multiplexer and demultiplexer respectively for the laser oscillation beam, but there is the new possibility that these are optical circulators, etc., which is a directional multiplexer-demultiplexer, and the efficiency is good. Because the laser oscillation beam is propagated in a direction reverse to that of the signal beam, is it possible to set the wavelength of the laser oscillation light irrespective of the wavelength of the signal beam, and degree of optionality of the components is increased, which is advantageous.

[0026] Fig. 6 shows the sixth structure of the present invention. The structure is analogous to that of Fig. 5 (the fifth structure of the present invention), but a excitation light source is newly installed, and the excitation light is supplemented by using the path of a laser ring. Therefore, it is possible to increase the total excitation light strength, and increase the saturation output of the optical amplifier.

[0027] Above, the first through sixth structures of the present invention have been shown, but below, in order to clarify the differences with the related technology, the structure of a typical example of the related technology and the present invention and the gain characteristics when using these structures are explained referring to the figures. The gain medium is an erbium-doped fiber (Er^{3+} doped fiber: EDF). The erbium doping concentration is 1000 ppm, and the unsaturated absorption coefficient of the signal beam at 1550 nm is 1 dB / m.

[0028] Fig. 11A and Fig. 11B show a first and second structure of a typical example of the related technology. Fig. 11A is a first typical example of the related technology wherein the excitation wavelength is 1.48 μm. The length of the EDF 11 - 6 is 50 m, the excitation light power is 100 mW, and the peak loss of the gain equalizer 11 - 3 is 10 dB or less. The wavelength dependency of the gain under these conditions is shown in Fig. 12A. The flattened-gain is 20 dB, and the flattened bandwidth is 30 nm (1535 ~ 1565 nm).

[0029] Fig. 11B is a second typical example of the related technology whose excitation wavelength is 1.55 μm. The length of the EDF 12 - 4 is 150 m, the excitation light power is 200 mW, and the peak loss of the gain

equalizer 11 - 3 is 10 dB or less. The wavelength dependency of the gain (the gain spectrum) under these conditions is shown in Fig. 12A. The flattened gain is 20 dB and the flattened. bandwidth is 40 nm (1570 ~ 1610nm).

[0030] Fig. 13 shows the structure of a typical example of the present invention. It is a two-stage amplifier structure, wherein the length of the pre-stage EDF 13 - 7 is 100 m, and the length of the post-stage EDF 13 - 11 is 70 m. In addition, the gain of the pre-stage EDF 13 - 7 is 25 dB, and the gain of the post-stage EDF 13 - 11 is 15 dB. The peak loss of the gain equalizer 13 - 4 disposed therebetween is 20 dB. The total gain spectrum is shown in Fig. 12B. The flattened-gain is 20 dB, and the flattened-bandwidth is 50 nm (1550 - 1600 nm).

[0031] As is clear by comparing the gain spectrum of the typical examples of the related technology and the present invention described above, by using the structure of the present invention, the flattened bandwidth is remarkably widened.

[0032] As described above, in the present invention the total gain is expanded without the influence of gain degradation due to laser oscillation and amplified spontaneous light emission by dividing the gain medium in two or three or more places and installing it, and at the same time, a bandwidth having a gain of a certain constant or greater has been increased in comparison with the related technology. In addition, by making the loss value of the gain equalizer large, it is possible to broaden the gain-flattened bandwidth in comparison to the related technology. Furthermore, because the structure installs the gain medium on post-stage of the gain equalizer, even if the loss value of the gain equalizer becomes large, it is possible to maintain the large saturation output of the optical amplifier, and it overcomes the drawback of the related technology that the saturation output of the optical amplifier is remarkably lowered when the loss value of the gain equalizer becomes large.

[First Embodiment]

[0033] Below, the first embodiment of the present invention is explained referring to the figures.

[0034] Fig. 14 is a block diagram showing an example of a structure of a wide bandwidth optical amplifier according to the first embodiment of the present invention.

[0035] In this embodiment, an erbium doped fiber (Er^{3+} doped fiber: EDF) is used as a gain medium, and has the structure of a two-stage amplifier. The concentration of the erbium dopant is 1000 ppm, and the unsaturated absorption coefficient of the signal beam at 1550 nm is 1 dB / m. The length of the pre-stage EDF 14 - 8 is 100m, and the length of the post-stage EDF 14 - 12 is 70 m. The excitation light sources 14 - 6 and 14 - 10 is a 1.53 μm semiconductor laser (LD), and the excitation light power is 100 mW. The excitation light and multiplexers 14 - 7 and 14 - 11 are an induction multilayer film filter, and the gain equalizer 14 - 4 is a split beam Fourier filter (Fourier filter). The peak loss of the gain equalizer (Fourier filter) 14 - 4 is 17 dB. The gain of the pre-stage EDF 14-8 is 25 dB, and the gain of the post-stage EDF 14-12 is 15 dB. Two optical isolator are installed in the pre-stage amplifiers and one optical isolator is installed in post-stage amplifier for preventing laser oscillation. Moreover, parameters, which makes flattened-gain bandwidth wide and is effective at the wide wavelength band of the gain medium, is set at the gain equalizer 14-4.

[0036] The gain spectrum of the first embodiment of the present invention is shown in Fig. 15. A flattened gain of 17 dB and a gain-flattened bandwidth of 50 nm are obtained. In addition, the saturation output with a multiple wavelength signal output (for example, 20 channels, or 100 channels) at 1.54 ~ 1.61 μm is 15 dBm, which is sufficiently high. However, the insertion loss of the multiplexers 14 - 7 and 14 - 11, optical isolators 14 - 9 and 14 - 13, and the gain equalizer (Fourier filter) 14-4 are each 1 dB.

[Second embodiment]

[0037] Next, the second embodiment of the present invention will be explained.

[0038] Fig. 16 is a block diagram showing an example of a structure of a wide bandwidth optical amplifier according to the second embodiment of the present invention.

[0039] The excitation light sources are different from those in Fig. 14 (the first embodiment). The excitation light sources 16 - 4 and 16 - 8 are LDs having a wavelength of 1.48 μm and an output power of 100 mW and the excitation light sources 16 - 6 and 16 - 10 are LDs having a wavelength of 1.55 μm, and an output optical power of 1 mW. The excitation light of 1.48 μm input into the EDFs 14 - 8 and 14 - 12 is absorbed by each EDF 14 - 8 and 14 - 12, and the 1.55 μm excitation light is amplified by each EDF 14-8 and 14-12. As a result, in the upper part of each EDF 14-8 and 14 - 12, a 1.48 μm excitation light power is dominant, while in the lower part, a 1.55 μm excitation light power is dominant. In total, it is possible to obtain the same gain as the first embodiment with the excitation light having 1.53 μm wavelength.

[0040] Furthermore, because the population inversion at the upper part was raised by the excitation light having 1.48 μm wavelength, the noise characteristics increased. Specifically, the noise index lowered. Fig. 17 shows the dependency of the noise index upon the signal beam wavelength in the second and first embodiments. It is clear that the noise index of the second embodiment has become lower.

[Third embodiment]

[0041] Next, the third embodiment of the present invention is explained.

[0042] Fig. 18 shows a block diagram of an example of the structure of a wide band optical amplifier according to the third embodiment of the present invention.

[0043] Compared to Fig. 14 (the first embodiment), the present embodiment further comprises a Raman amplifier. The transmission fiber (silica fiber) 18 - 4 is excited by an excitation light source (LD) with a wavelength of 1.51 μm and an output optical power of 200 mW. The transmission fiber 18 - 4 is a 60 km dispersion-shifted fiber. The Raman gain at 1.61 μm is 10 dB. Fig. 19 shows the gain spectrum according to the present embodiment. Compared to the first embodiment, the flattened gain is raised 5 dB and the gain bandwidth is raised 25 nm.

[0044] Moreover, an optical circulator, which is a directional coupler, can be used as a multiplexer 18 - 5. The reason is that because the directions of the excitation wavelength (1.51 μm) and the signal beam are different with respect to the transmission fiber, the multiplexing of light by an optical circulator can be easily carried out. In addition, compared to using a wavelength division multiplexing coupler as a multiplexer 18 - 5, by using an optical circulator, it is possible to amplify a signal beam near the excitation wavelength, and it is also possible to broaden the bandwidth which optically amplifies.

[Fourth embodiment]

[0045] Next the fourth embodiment of the present invention will be explained.

[0046] Fig. 20 is a block diagram showing an example of a structure of a wide bandwidth optical amplifier according to the fourth embodiment of the present invention.

[0047] Compared to Fig. 16 (the second embodiment), the present embodiment has the structure of the excitation unit of the EDF in the pre-stage and post-stage. Therefore, in Fig. 20, only the pre-stage amplifier 20 - 1 is shown. The structure of the post-stage (not shown) is the same as the structure of the pre-stage amplifier 20 - 1. The excitation light source 16 - 4 is an LD with a wavelength of 1.48 μm and an output light power 100 mW. Instead of using an LD with a wavelength of 1.55 μm, a high power laser oscillation beam with a wavelength of 1.55 μm is oscillated in the ring laser. A ring laser comprises EDF 14 - 8, ring laser multiplexer (multiplexers 20 - 2 and 20 - 3), a narrow bandwidth transmission optical filter 20 - 6, a tunable attenuator 20 - 5, and an optical isolator 20 - 4. The multiplexers 20 - 2 and 20 - 3 can use wavelength division multiplex coupler which only multiplexes and demultiplexes a laser oscillator optical wavelength in a narrow bandwidth. The obtained amplification characteristics are the same as those in the second embodiment. In the present structure, because there is only one excitation light source (LD), it has the advantages that the structure is simple and stable.

[Fifth embodiment]

[0048] Next, the fifth embodiment of the present invention is explained.

[0049] Fig. 21 is a block diagram showing an example of a structure of a wide bandwidth optical amplifier according to the fifth embodiment of the present invention.

[0050] Compared to Fig. 20 (fourth embodiment), the present embodiment has optical circulators 21 - 2 and 21 - 3 in the ring laser instead of an optical isolator 20 - 4 and ring laser multiplexers (multiplexers 20 - 2 and 20 - 3). It is advantageous to use the optical circulators 21 - 2 and 21 - 3, because the number of optical parts is decreased and the structure is simplified.

[Sixth embodiment]

[0051] Next, the sixth embodiment of the present invention is explained.

[0052] Fig. 22 is a block diagram showing an example of a structure of a wide bandwidth optical amplifier according to the sixth embodiment of the present invention.

[0053] Compared to Fig. 21 (the filth embodiment), the present embodiment has one more excitation light source in the ring laser loop. In this manner, there is the advantage that the total excitation optical power is increased without degradation of the signal beam gain, and the signal beam saturation output can be increased.

[0054] Moreover, in the above-described first through sixth embodiments, an example was explained wherein the amplifier has two stages, and a gain equalizer is disposed therebetween, but in the amplifiers explained in each embodiment, it is possible to provide N stages (N being an integer equal to or greater than 2), and provide a gain equalizer between each of the amplifiers.

[0055] Above, the embodiments of the present invention are explained in detail referring to the figures, but a concrete structure is not limited to these embodiments, and changes in design within the scope not departing from the spirit of the invention are included in this invention.

[0056] Above, according to the present invention, in comparison with the related technology, there are the effects that the gain flattened bandwidth is wide, and it is possible to realize a high saturation output, low noise optical amplifier.

[0057] Next, an optical amplifier provided with a Raman amplifier using a high nonlinear fiber or a dispersion compensation fiber as a Raman amplifier medium will be explained in the seventh through thirteenth embodiments.

[Seventh embodiment]

[0058] First, referring to Fig. 29, the seventh embodiment of the optical amplifier will be explained. Moreover, this seventh embodiment relates to a most basic structure of an optical amplifier provided with a Raman amplifier using a high nonlinear fiber as a Raman amplifier medium. As shown in this figure, the optical amplifier A of the present embodiment comprises a Raman amplifier A1 and a rare-earth doped fiber amplifier A2. In the optical amplifier A structured in this manner, a transmission fiber B1 (transmission path) for inputting an optical signal and a transmission fiber B2 (transmission path) for outputting an amplified optical signal are connected together.

[0059] In addition, the above-described Raman amplifier A1 comprises a nonlinear fiber a1 which is the Raman amplifying medium, an excitation light source 2a which generates an excitation light for exciting the high nonlinear fiber a1, and a multiplexer a3. To one end of the high nonlinear fiber a1, the above transmission fiber B1 is connected, and the optical signal is incident thereupon, and to the other end the multiplexer a3 is connected so that the excitation light supplied from the excitation light source a2 is incident thereupon.

[0060] That is, in contrast to the direction of incidence of the optical signal, the multiplexer a3 makes the excitation light incident on the high nonlinear fiber a1 from the opposite direction, and at the same time, the optical signal amplified by the high nonlinear fiber a1 is output to the rare-earth doped fiber amplifier A2. This rare-earth doped fiber amplifier A2, as described above, can provide an optical amplifying action by reflecting the excitation light into the rare-earth doped fiber, and can provide a gain flattening means such as a gain equalizer.

[0061] When structuring an optical amplifier A from a Raman amplifier A1 and a rare-earth doped fiber amplifier A2 in this manner, by adjusting the Raman gain of the Raman amplifier A1 for offsetting the decrease in the gain spectrum at the long wavelength region in the rare-earth doped fiber A2, it is possible that the gain realizes a flattened region over a wide bandwidth.

[0062] In this manner, in using a high nonlinear fiber a1 as a Raman amplifier medium, the present embodiment is very different from the optical amplifier which uses an optical transmission fiber shown in the third embodiment as the Raman amplifier medium. Generally, a high nonlinear fiber has a mode radius which is small in comparison to the transmission fiber usually used, and in addition, because the concentration of the dopant is high, the efficiency of the nonlinear effects of the light are high, and thus it is possible to carry out highly efficient Raman amplification even in a comparatively short fiber length and low excitation light power. By such a high nonlinear fiber, a rate of Raman amplification proportionate to the square of the core diameter and the concentration of the dopant can be obtained. Therefore, because it is possible, for example, to have a fiber length of several kilometers with in-line optical amplifiers, it is possible to construct the lumped parameter optical amplifier, and at the same time, it is possible to construct the optical amplifier having an efficient Raman amplifier.

[0063] For example, as typical values for the parameters of the Raman amplifier A1 structured from this kind of high nonlinear fiber a1, the mode diameter and fiber length of the high nonlinear fiber a1 are respectively 4 μm and 1 km, and the power of the excitation light from the excitation source a2, which is a 1.51 μm excitation semiconductor laser, is 200 mW.

[Eighth embodiment]

[0064] Referring to Fig. 30, the eight embodiment of the optical amplifier of the present invention will be explained. Moreover, this embodiment relates to a variation of the structure of the rare-earth doped fiber amplifier A in the above-described seventh embodiment. Therefore, the Raman amplifier is the same as the above-described Raman amplifier A1, and thus the explanation of identical reference numbers is omitted.

[0065] As shown in the figure, the rare-earth doped fiber amplifier A3 in the present embodiment comprises a pre-stage amplifier 1, a post-stage amplifier 2, and a Fourier filter (split beam Fourier filter) 3 interposed therebetween. In addition, the pre-stage amplifier 1 comprises isolators 1a and 1d, a multiplexer 1b, a rare-earth doped fiber 1c, and an excitation light source 1e (a semiconductor laser); the post-stage amplifier 2 comprises a multiplexer 2a, a rare-earth doped fiber 2b, an isolator 2c, and an excitation light source 2d (a semiconductor laser).

[0066] The optical signal output from the Raman amplifier A1 is incident on the isolator 1a, and output to the Fourier filter 3 from the isolator 1d via the multiplexer 1b and then the rare-earth doped fiber 1c. In addition, the excitation light output from the excitation light source 1e is incident on the rare-earth doped fiber 1c via the multiplexer 1b. The Fourier filter 3 acts as a gain equalizing means, and the optical signal input from the pre-stage amplifier 1 is gain-equalized and output to the post-stage amplifier 2.

[0067] In addition, the optical signal emitted from Fourier filter 3 in this manner is incident on the multiplexer 2a of the post-stage amplifier 2, and emitted from the isolator 2c via the rare-earth doped fiber 2b. Additionally, in the rare-earth doped fiber 2b, the excitation light generated in the excitation light source 2d is output via the multiplexer 2a.

[0068] According to the above-described rare-earth doped fiber amplifier A3, the optical signal, which is Raman-amplified by the high nonlinear fiber a1 in the Raman amplifier A1, is optically amplified by the rare-earth doped fiber 1c, and then it is gain-equalized by the Fourier filter 6, and it is further amplified by the rare-

earth doped fiber 2b.

[0069] In the present embodiment, because it is possible to construct the optical amplifier from the above-described comparatively short length high nonlinear fiber a1 and the amplifier which can carry out Raman amplification efficiently with comparatively low power excitation light and has the characteristic of a lumped-parameter, the optical amplifier can be provided with the above-described construction of a rare-earth doped fiber amplifier A3 as a post-amplifier, which is impossible in conventional construction.

[0070] Moreover, in Fig. 30, the rare-earth doped fiber amplifier A3 can be constructed from N amplifiers, explained in the first through sixth embodiments, and (N - 1) gain equalizers provided therebetween (N being an integer equal to or greater than 2).

[0071] Here, in the present embodiment, the excitation light for Raman amplification is multiplexed using a multiplexer a3, but in place of the multiplexer a3 a directional coupler such as a light circulator can also be used. In this case, the isolator 1a of the pre-stage amplifier 1 is unnecessary, and it is possible to decrease the loss of the optical signal in this isolator 1a.

[0072] Moreover, the construction of the gain-flattened rare-earth doped fiber amplifier included in the present embodiment is described in detail in a Citation (H. Masuda, et al., Electron. Lett., Vol. 33, pp. 1070-1072, 1997).

[Ninth embodiment]

[0073] Next, referring to Fig. 31, the ninth embodiment of the optical amplifier according to the present invention is explained. This embodiment alters the Raman amplifier A1 in the above-described eighth embodiment to Raman amplifier A4, and other parts of the construction are the same at that in the eight embodiment. That is, because excitation light is also input from the front of a high nonlinear fiber a1 (the input side of the optical signal), this Raman amplifier A4 is provided with a multiplexer a4 at the input end of the optical signal, and excitation light emitted from the excitation light source a5 (a second light emission source) via the multiplexer a4 is supplied to the high nonlinear fiber a1 from the front.

[0074] By adopting this kind of structure, in the case when, for example, the power of the excitation light source a2 is made equal to the power of the excitation optical source a5, for the above-described second embodiment, it is possible to supply twice the power of the excitation light a5 to the high nonlinear fiber a1, so in a state of comparative suppression of the power of each of the light sources a2 and a5, it is possible to Raman amplify the optical signal with even more efficiency. Moreover, of course, it is not necessary that the power of the excitation light source a2 and the power of the excitation light source a5 be equal.

[Tenth embodiment]

[0075] Fig. 32 shows the construction of the tenth embodiment of the optical amplifier of the present invention.

[0076] The present embodiment relates to a variation of the structure of the Raman amplifiers for each of the above-described embodiments. That is, in contrast to the Raman amplifier shown in the above Fig. 29, the Raman amplifier A5 of the present embodiment is characterized in being provided with an isolator a6 on the input terminal of the optical signal, that is, the input terminal of the high nonlinear fiber a1. By adopting this structure, it is possible to stop the leaking of the excitation light transiting the high nonlinear fiber a1 into the transmission fiber.

[Eleventh embodiment]

[0077] Fig. 33 shows the construction of the eleventh embodiment of the optical amplifier of the present invention.

[0078] This embodiment is also related to a variation of the construction of a Raman amplifier similar to that in the above-described tenth embodiment. That is, in contrast with the Raman amplifier A1 shown in the above Fig. 29, the Raman amplifier A6 of the present embodiment is characterized in being provided with a multiplexer a7 at the input terminal of the optical signal, and additionally provided with a rare-earth doped fiber a8 between the multiplexer a7 and the high nonlinear fiber a1. Furthermore, it is characterized in being provided with an excitation light source 39 which supplies excitation light to the high nonlinear fiber a1 and the rare-earth doped fiber a8 via the multiplexer a7.

[0079] Fig. 28 is the gain spectrum when a silica fiber transmission path is used as a Raman amplifier. In this case, because a large Raman gain is obtained in the short-wave region of the gain bandwidth, it is possible that the noise characteristics degrades in the Raman amplifier A1. In this embodiment, because the signal beam is Raman amplified by the high nonlinear fiber a1 after being amplified by rare-earth doped fiber a8, it is possible to prevent degradation of the noise characteristics in the short-wave region in the above gain bandwidth.

[Twelfth embodiment]

[0080] Fig. 34 shows the construction of the twelfth embodiment of the optical amplifier of the present invention.

[0081] This embodiment also relates to a variation of the structure of the optical amplifier similar to that in the above tenth and eleventh embodiments. That is, in contrast to the construction of the Raman amplifier A1 shown in the above figure, the Raman amplifier A7 of the present embodiment is characterized is using a dis-

persion compensation fiber 10 in place of the high nonlinear fiber a1.

[0082] Generally, because a dispersion compensation fiber is, like a high nonlinear fiber, etc., characterized in having a small core diameter and a high concentration of dopant, it is possible to use one as a Raman amplifying medium. By using this kind of dispersion compensation fiber, it is possible to compensate transmission path dispersion which accumulates in during signal propagation. In this state, a dispersion compensation of about -200~+200 ps / nm / dB is possible by using a dispersion compensation fiber, it can also sufficiently compensate the accumulated dispersion not only in the transmission system using a dispersion-shifted fiber as a transmission path, but also in a transmission system using a single mode fiber as a transmission path.

[0083] Additionally, in the case of a post-amplifier structure, the input optical power to the optical amplifier becomes large, and the multi-wavelength signal beam of the adjacent zero-dispersion wavelength can produce cross-talk between adjacent channels due to the influence of nonlinear effects such as four-wave mixing, but by using the dispersion compensation fiber a 10 having a high dispersion such as in the present embodiment, it is possible to suppress this kind of cross-talk.

[Thirteenth embodiment]

[0084] Next, referring to Fig. 35, the thirteenth embodiment of the optical amplifier according to the present invention will be explained. The present embodiment relates to a variation of the structure of the Raman amplifier in the above-described twelfth embodiment. That is, in place of the above-described dispersion compensation fiber a10, the Raman amplifier A8 of the present embodiment is characterized in applying a dispersion compensation fiber a11 wherein the dispersion slope relates inversely to the dispersion slope of the transmission fiber.

[0085] In this manner, by the dispersion slope of the dispersion compensation fiber a11 inversely relating to that of the transmission fiber, it is possible to compensate not only the transmission path dispersion but also high order dispersion (wavelength dependent dispersion; by this, the difference in accumulated dispersion value between signal channels with different wavelengths is occurred).

[0086] Next, the embodiments of the transmission systems using the optical amplifier shown in the above-described twelfth and thirteenth embodiments will be explained in referring to the figures. That is, the following embodiment of the optical transmission system is related to an optical amplifier using a dispersion compensation fiber.

[First embodiment of an optical transmission system]

[0087] First, referring to Figs. 36A and 36B, the first embodiment of an optical transmission system will be explained. As shown in Fig. 36A, the optical transmission system of the present embodiment comprises a transmitter 9, a dispersion-shifted fiber 10 having a zero-dispersion wavelength in the 1.5 μm band, an optical amplifier 11, and a receiver 12.

[0088] The optical amplifier 11 is the amplifier of the above twelfth and thirteenth embodiments using a dispersion compensation fibers (a10 or a11) as a Raman amplifying medium. The present optical transmission system is constructed with the transmitter 9 and the receiver 12 connected by the dispersion-shifted fiber 10, and optical amplifiers 11 inserted at each specified repeater interval of the dispersion-shifted fiber 10. Here, each parameter of the dispersion compensation fibers (a10 or a11) are set so as to compensate the dispersion of the transmission path, in the present embodiment, the dispersion-shifted fiber 10.

[0089] For example, as shown in Fig. 36B, in the case of a dispersion value of the above-described dispersion-shifted fiber 10 of a certain signal beam wavelength being 2 ps / nm / km, and the repeater interval being 100 km, dispersion compensation is possible by setting the dispersion value of the dispersion compensation fibers (a10 or a11) in the optical amplifier 11 and the fiber length to -100 ps / nm / km and 2 km, respectively. That is, as shown in the figure, because the area of the transmission by the dispersion-shifted fiber 10 and the area of the transmission of the dispersion-shifted fibers (a10 and a11) in the optical amplifier 11 are equal, dispersion is compensated.

[0090] In addition, as described above, by making the dispersion slope of the dispersion-shifted fiber 10 inverse that of the dispersion compensation fibers (a10 and a11), high order dispersion compensation is possible.

[Second embodiment of the optical transmission system]

[0091] Next, referring to Figs. 37A and B, the second embodiment of the optical transmission system of the present invention will be explained. This embodiment, as shown in Fig. 37A, is characterized in using a single mode fiber 13 having a zero dispersion wavelength in the 1.3 μm wavelength band as a transmission path instead of the dispersion-shifted fiber 10 of the above first embodiment. In addition, each parameter of dispersions compensation fibers (a10 and a11) are set so as to compensate the dispersion of the transmission path, that is to say, the single mode fiber 13.

[0092] As shown in Fig. 37B, when, for example, the dispersion value of a single mode fiber is at a certain signal beam wavelength is 15 ps / nm / km, and the repeater interval is 100 km, by setting the dispersion value of the dispersion compensation fibers (a10 and a11) in the optical amplifier 11 and the fiber length to 150 pa /nm/ km and 10 km respectively as shown in the

figure, the dispersion is compensated because the area of the transmission by the single mode fiber 13 and the area of the transmission of the dispersion compensation fibers (a10 and a11) become the identical. In addition, as described above, by making an inverse relationship between the dispersion slope of the single mode fiber 13 and the dispersion slope of the dispersion compensation fibers (a10 and a11) in the optical amplifier 11, high order dispersion compensation is possible.

[0093] Moreover, the present invention shown in the seventh through thirteenth embodiments and the two embodiments of the optical transmission system are not limited to the embodiments described above, and the following alterations can be conceived:

(1) In place of the multiplexer a3 of each embodiment, a bi-directional coupler such as an optical circulator can be used. In this case, because it is not necessary to insert an isolator at the input terminal of the rare-earth doped fiber amplifier, it is possible to reduce the loss of the optical signal in the isolator.

(2) Even in the case of using a dispersion compensation fiber as a Raman amplification medium, the excitation light is input at the front and back of the Raman amplifying medium like the above-described ninth embodiment of the optical amplifier.

(3) Even when using a dispersion compensation fiber as a Raman amplifying medium, an isolator can be provided at the input terminal of the optical signal of the dispersion compensation fiber like the above-described tenth embodiment of the optical amplifier.

(4) Even when using a dispersion compensation fiber as a Raman amplifying medium, rare-earth doped fibers can be arranged serially with dispersion compensation fibers like the eleventh embodiment.

(5) In addition to the above, in each embodiment of the above-described optical amplifier using a high nonlinear fiber as a Raman amplifying medium, it is applicable to use a dispersion compensation fiber as a Raman amplifying medium, and it is conceivable to combine each embodiment of the optical amplifier using a dispersion compensation fiber with a means of each above-described embodiment using a high nonlinear fiber. In addition, for an optical amplifier making up by an optical transmission system as well, combining an optical amplifier of each of the above-described embodiments using a dispersion compensation fiber with a means of an optical amplifier of each of the above-described embodiments using a high nonlinear fiber can be conceived.

[0094] As explained above, the following effects can be obtained from an optical amplifier according to the above seventh through thirteenth embodiments and an optical transmission system using it:

(1) By combining a Raman amplifier using a Raman amplifying medium and a rare-earth doped fiber amplifier using a rare-earth doped fiber as an amplifying medium, in an optical amplifier carrying out wide bandwidth optical amplification, because a high nonlinear fiber or a dispersion compensation fiber are applied as a Raman amplifying medium, a lumped parameter optical amplifier with a flattened gain bandwidth and a wide bandwidth can be constructed.

(2) In addition, when using a dispersion compensation fiber as a Raman amplifying medium, because it is possible to compensate the dispersion of a transmission path, it is possible to realize large-capacity wavelength division multiplex optical transmission.

[0095] As explained above, by the present invention, it is possible to greatly broaden the gain bandwidth of an amplifier. Thus, it is possible to increase the number of signal beam channels and the transmission capacity, and it is possible to realize the high degree and economic wavelength division multiplexing system.

[0096] In addition, the present invention can be widely used not only in a ground-based trunk transmission system but also in a subscriber system, the optical transmission of wavelength division multiplexing of submarine repeater transmission path system, and so on.

Claims

1. An optical amplifier, comprising:

   N amplifiers including a gain medium using a rare-earth doped fiber as a long length gain medium, which are divided into N stages, N being an integer equal to or greater than 2, and an excitation light source which outputs excitation light whose effective excitation wavelength of said gain medium is 1.53 μm; and

   (N - 1) gain equalizers which are installed between said N amplifiers respectively, and are effective over the wide wavelength band of said gain medium.

2. An optical amplifier according to Claim 1, wherein said excitation light comprises a plurality of excitation lights of different excitation wavelengths.

3. An optical amplifier according to Claim 1, wherein said rare-earth doped fiber is an erbium doped fiber.

4. An optical amplifier according to Claim 1, further comprising:

an excitation light source which sends Raman amplifying excitation light to a silica fiber transmission path connecting said optical amplifiers.

5. An optical amplifier according to Claim 4, wherein said optical amplifier further comprises a directional coupler on the input side, and

said Raman amplifying excitation light is sent to a Raman amplifying medium via said directional coupler.

6. An optical amplifier according to Claim 5, wherein said Raman amplifying excitation light has a wavelength which makes the amplification band of said optical amplifier wide.

7. An optical amplifier according to Claim 6, wherein:

said rare-earth doped fiber is an erbium doped fiber; and
the wavelength of the Raman amplification excitation light is in the range of 1.49 to 1.53 μm.

8. An optical amplifier according to Claim 2, wherein said amplifier is provided with a ring construction in which a second desired excitation light differing from a first excitation optical wavelength from said excitation optical source oscillates in the same direction as the signal beam.

9. An optical amplifier according to Claim 8, wherein said ring construction includes a WDM coupler which multiplexes and demultiplexes the second excitation light in a signal beam and a light amplified by said gain medium.

10. An optical amplifier according to Claim 2, wherein said amplifier is provided with a ring construction wherein a desired second excitation light which is different from a first excitation light wavelength by said excitation light source oscillates in the opposite direction of the signal beam.

11. An optical amplifier according to Claim 10, wherein said ring structure includes a directional coupler which multiplexes said second excitation light in a signal beam and a light by amplified by said gain medium.

12. An optical amplifier according to Claim 10, wherein said ring construction further comprises:

a second excitation light source which generates an excitation light which is the same frequency of said first excitation light; and
a multiplexer which optically multiplexes excitation light from said second excitation light source in said ring construction.

13. An optical amplifier, comprising:

a Raman amplifier which is provided with an internal Raman amplifier medium and carries out Raman amplification by said Raman amplification medium; and
a rare-earth doped fiber amplifier in which a rare-earth doped fiber is used as an amplification medium.

14. An optical amplifier according to Claim 13, wherein said Raman amplifier medium is a high nonlinear fiber.

15. An optical amplifier according to Claim 13, wherein said Raman amplifying medium is a dispersion compensation fiber.

16. An optical amplifier according to Claim 14, wherein said Raman amplifier comprises:

a high nonlinear fiber into one terminal of which a optical signal is input via a transmission fiber;
an excitation light source which generates an excitation light; and
a multiplexer which is connected to the other terminal of said high nonlinear fiber; and which makes the excitation light input into said high nonlinear fiber, and outputs an optical signal input form said high nonlinear fiber into said rare-earth doped fiber amplifier.

17. An optical amplifier according to Claim 15, wherein said Raman amplifier comprises:

a dispersion compensation fiber into one terminal of which an optical signal is input via a transmission fiber;
an excitation light source which generates an excitation light; and
a multiplexer which is connected to the other terminal of said dispersion compensation fiber, and which makes the excitation light input into said dispersion compensation fiber, and outputs an optical signal input form said high nonlinear fiber into said rare-earth doped fiber amplifier.

18. An optical amplifier according to Claim 16, wherein said Raman amplifier further comprises:

a second excitation light source which generates excitation light; and
a second multiplexer which makes the excitation light input into said one terminal of the

Raman amplifying medium.

19. An optical amplifier according to Claim 18, wherein said Raman amplifier comprises:

a rare-earth doped fiber inserted between said second multiplexer and said Raman amplifying medium.

20. An optical amplifier according to Claim 16, wherein said Raman amplifier further comprises:

an isolator which is installed at the terminal of said Raman amplifying medium and prevents leakage of the excitation light into the transmission fiber.

21. An optical amplifier according to Claim 13, wherein said rare-earth doped amplifier comprises:

a pre-stage amplifier which uses a rare-earth doped fiber as an amplifying medium;
a post-stage amplifier which similarly uses a rare-earth doped fiber as an amplifying medium; and
a gain equalizing means inserted between said post-stage amplifier and said pre-stage amplifier.

22. An optical amplifier according to Claim 15, wherein the dispersion slope of said dispersion compensation fiber has a reverse relationship with the dispersion slope of the transmission fiber.

23. An optical amplifier according to Claim 16, wherein said Raman amplifier uses a directional coupler instead of said multiplexer.

24. An optical amplifier according to Claim 15, wherein said dispersion compensation fiber has parameters which are set to compensate a dispersion-shifted fiber which is a transmission path.

25. An optical transmission system, comprising:

a transmitter which transmits an optical signal;
a transmission path using a dispersion-shifted fiber;
a receiver which receives an optical signal; and
an optical fiber, comprising a Raman amplifier which carries out Raman amplification by a dispersion compensation fiber whose parameters are set to compensate the dispersion of said transmission path, and a rare-earth doped fiber amplifier which uses a rare-earth doped fiber as an amplifier medium.

26. An optical amplifier according to Claim 25, wherein said Raman amplifier comprises:

a dispersion compensation fiber into one terminal of which an optical signal is input via a transmission fiber;
an excitation light source which generates an excitation light; and
a multiplexer which is connected to the other end of said dispersion compensation fiber, and which makes the excitation light input into said dispersion compensation fiber, and outputs an optical signal input from said dispersion compensation fiber into said rare-earth doped fiber amplifier.

27. An optical amplifier according to Claim 26, wherein said Raman amplifier further comprises:

a second excitation light source which generates excitation light;
a second multiplexer which makes the excitation light input into said one terminal of the Raman amplifying medium.

28. An optical amplifier according to Claim 27, wherein said Raman amplifier further comprises:

a rare-earth doped fiber inserted between said second multiplexer and said Raman amplifying medium.

29. An optical amplifier according to Claim 26, wherein said Raman amplifier further comprises:

an isolator which is installed at the terminal of said Raman amplifying medium, and prevents leakage of the excitation light into the transmission fiber.

30. An optical fiber according to Claim 25, wherein said rare-earth doped fiber comprises:

a pre-stage amplifier which uses a rare-earth doped fiber as an amplifying medium;
a post-stage amplifier which similarly uses a rare-earth doped fiber as an amplifying medium; and
a gain equalizing means inserted between the post-stage amplifier and the pre-stage amplifier.

31. An optical fiber according to Claim 26, wherein said Raman amplifier uses a directional coupler instead of said multiplexer.

32. An optical transmission system comprising:

a transmitter which transmits an optical signal;

a transmission path using a single mode fiber; and

an optical amplifier; comprising a Raman amplifier which carries out Raman amplification by a dispersion compensation fiber whose parameters are set to compensate the dispersion of said transmission path, and a rare-earth doped fiber amplifier which uses a rare-earth doped fiber as an amplifier medium.

33. An optical amplifier according to Claim 32, wherein said Raman amplifier comprises:

a dispersion compensation fiber into one terminal of which an optical signal is input via a transmission fiber;

an excitation light source which generates excitation light; and

a multiplexer which is connected to the other terminal of said dispersion compensation fiber, and which makes the excitation signal input into said dispersion compensation fiber, and output an optical signal input from said dispersion compensation fiber into said rare-earth doped fiber amplifier.

34. An optical amplifier according to Claim 33, wherein said Raman amplifier further comprises:

a second excitation light source which generates excitation light; and

a second multiplexer which makes the excitation light input into said one terminal to a Raman amplifying medium..

35. An optical amplifier according to Claim 34, wherein said Raman amplifier further comprises:

a rare-earth doped fiber inserted between said second multiples and said Raman amplifying medium.

36. An optical amplifier according to Claim 33, wherein said Raman amplifier further comprises:

an isolator which is installed at the terminal of said Raman amplifying medium and prevents leakage of the excitation light into the transmission fiber.

37. An optical amplifier according to Claim 32, wherein said rare-earth doped fiber amplifier comprises:

a pre-stage amplifier which uses a rare-earth doped fiber as an amplifying medium;

a post-state amplifier which similarly uses a rare-earth doped fiber as an amplifying medium; and

a gain equalizing means inserted between the post-stage amplifier and the pre-stage amplifier.

38. An optical amplifier according to Claim 33, wherein said Raman amplifier uses a directional coupler instead of said multiplexer.

FIG.1

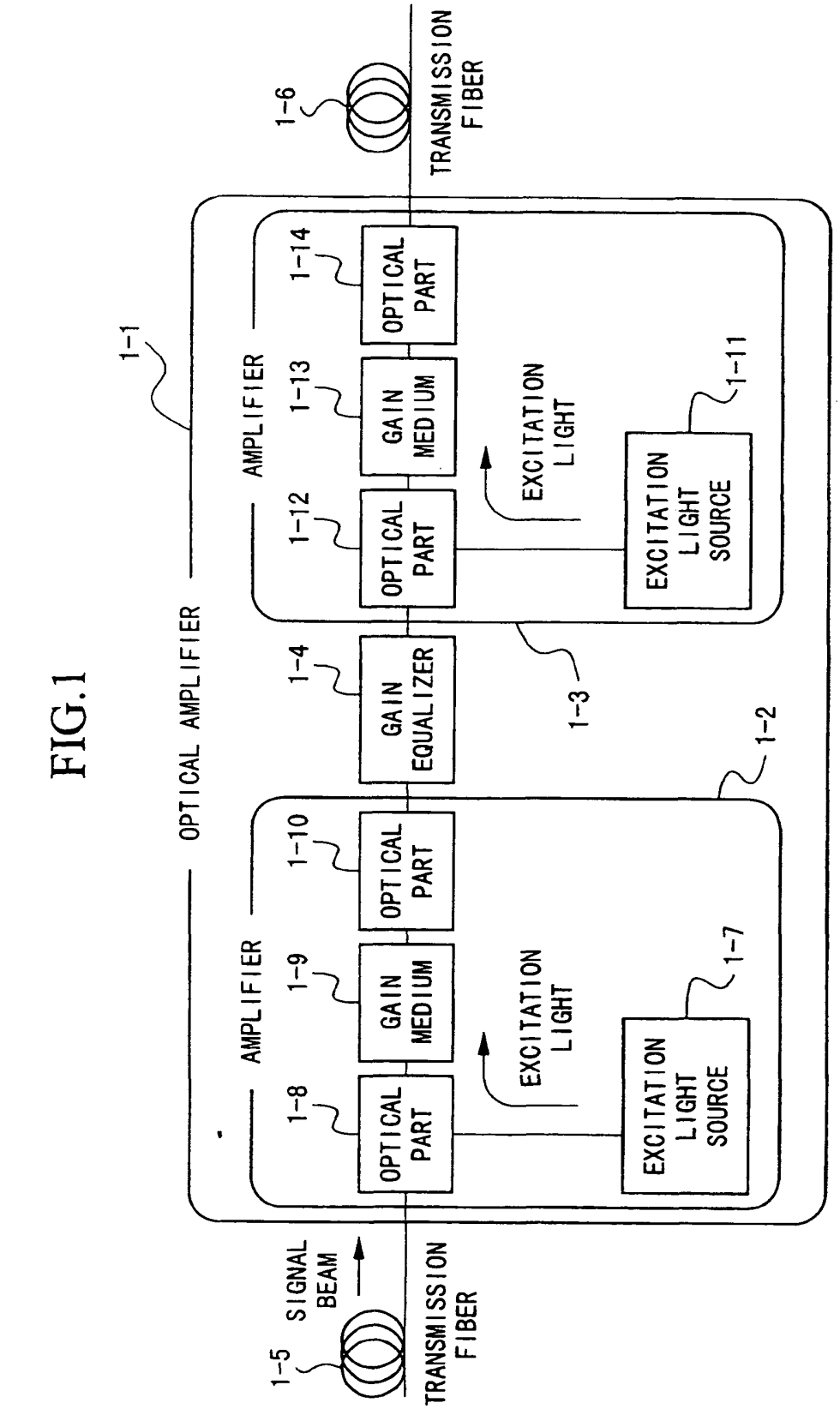

1-1
OPTICAL AMPLIFIER
1-5
SIGNAL BEAM
TRANSMISSION FIBER
1-6
TRANSMISSION FIBER
AMPLIFIER
1-8
OPTICAL PART
1-9
GAIN MEDIUM
1-10
OPTICAL PART
EXCITATION LIGHT
1-7
EXCITATION LIGHT SOURCE
1-2
1-4
GAIN EQUALIZER
1-3
AMPLIFIER
1-12
OPTICAL PART
1-13
GAIN MEDIUM
1-14
OPTICAL PART
EXCITATION LIGHT
1-11
EXCITATION LIGHT SOURCE

FIG.2

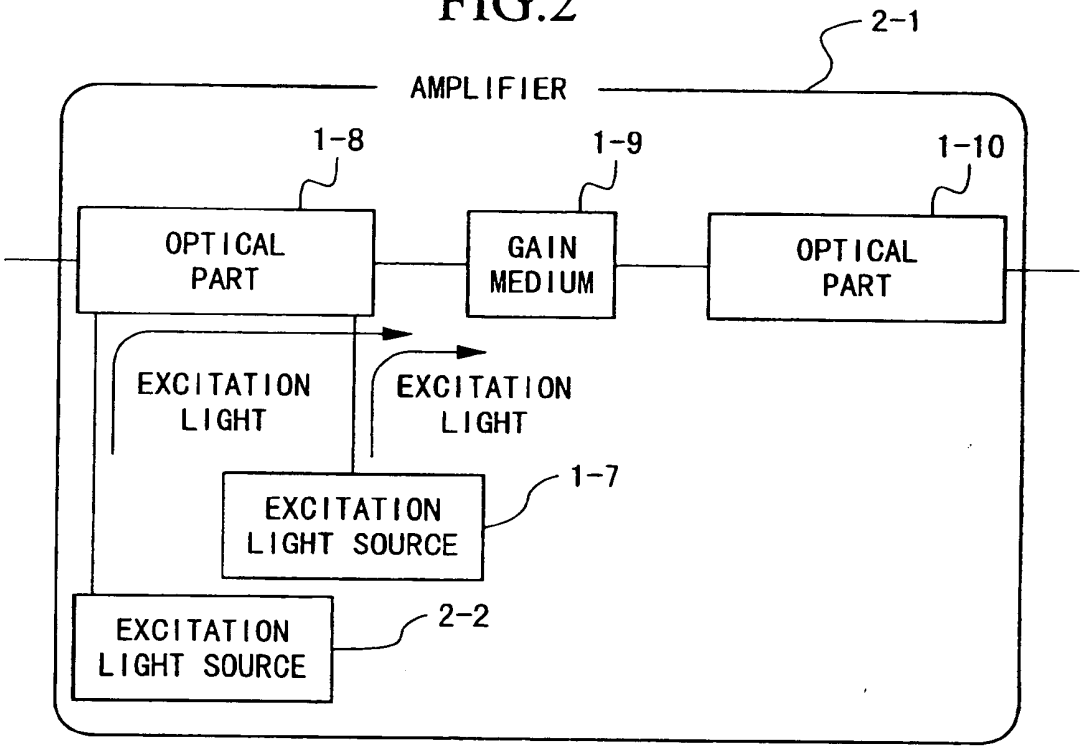

2-1
AMPLIFIER
1-8
1-9
1-10
OPTICAL PART
GAIN MEDIUM
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT
1-7
EXCITATION LIGHT SOURCE
2-2
EXCITATION LIGHT SOURCE

FIG.4

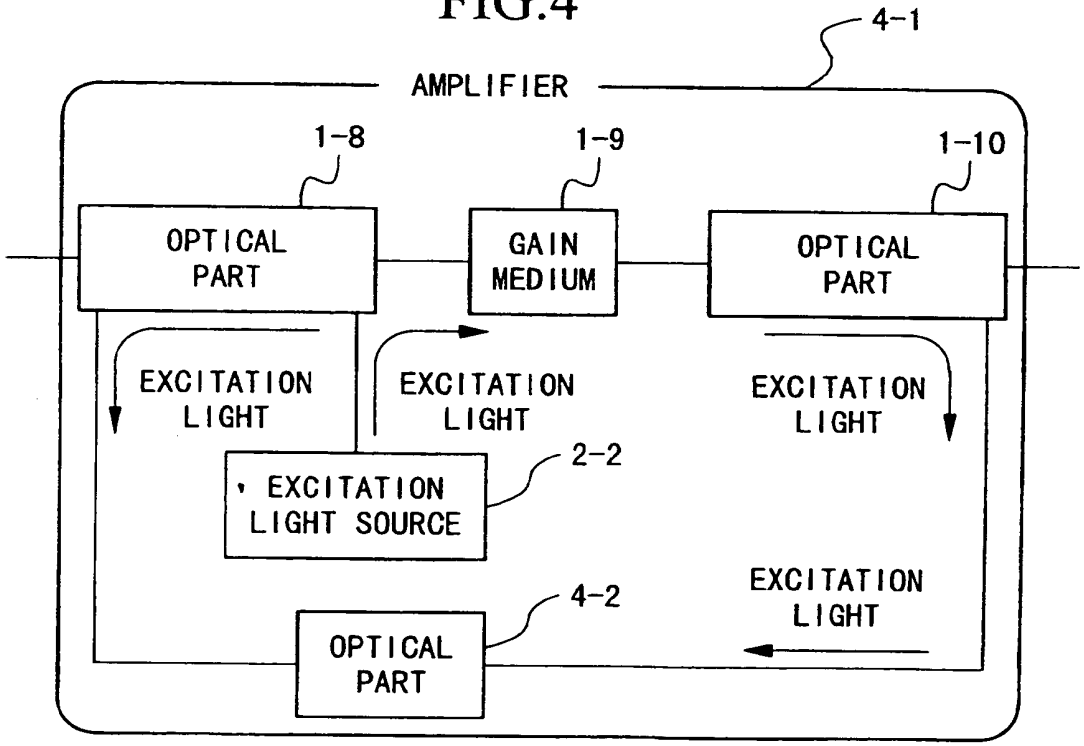

4-1
AMPLIFIER
1-8
1-9
1-10
OPTICAL PART
GAIN MEDIUM
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
2-2
EXCITATION LIGHT SOURCE
EXCITATION LIGHT
4-2
OPTICAL PART

FIG.3

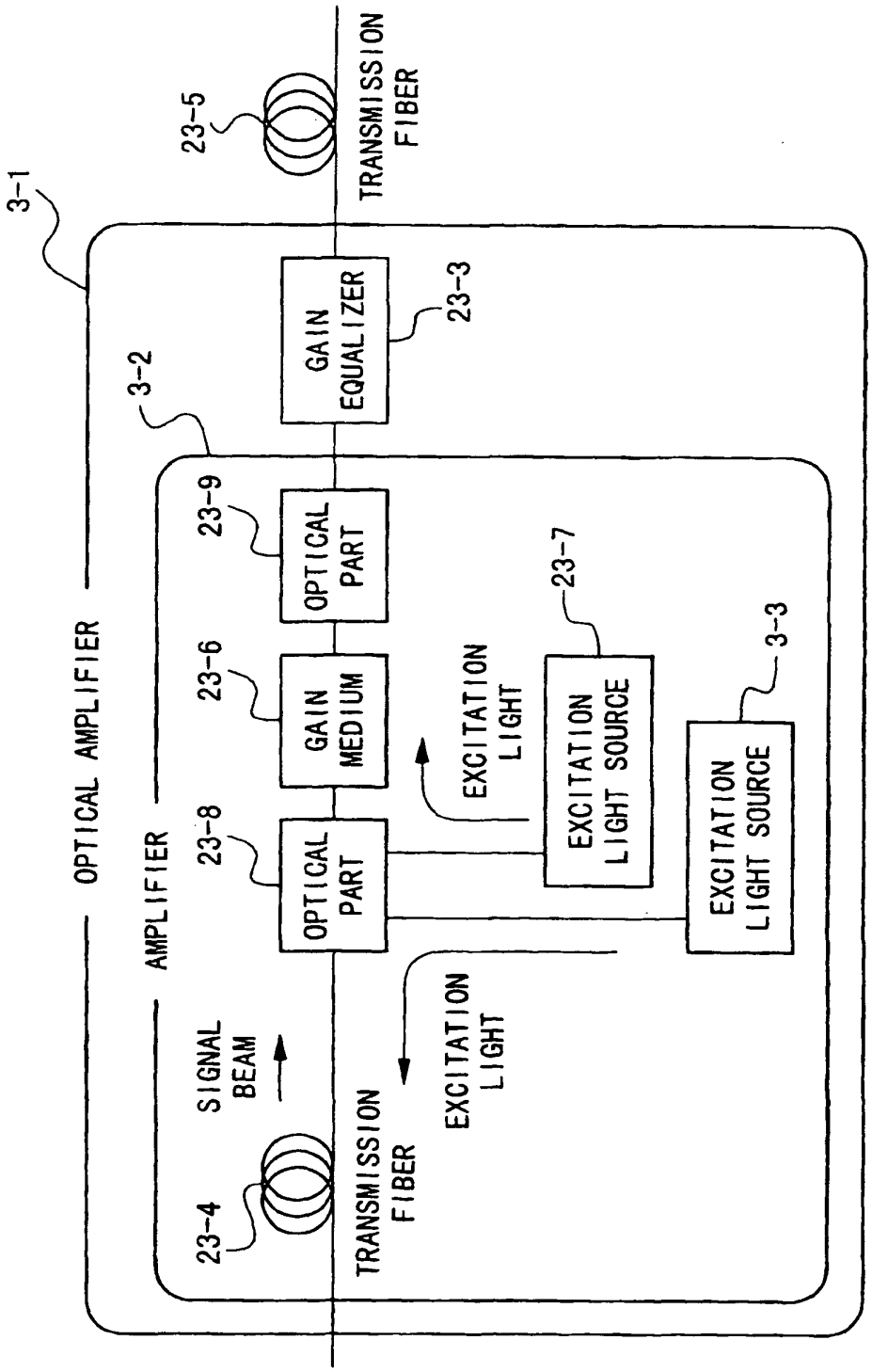

3-1
OPTICAL AMPLIFIER
AMPLIFIER
3-2
23-5
TRANSMISSION FIBER
GAIN EQUALIZER
23-3
23-9
OPTICAL PART
23-6
GAIN MEDIUM
23-8
OPTICAL PART
EXCITATION LIGHT
23-7
EXCITATION LIGHT SOURCE
3-3
EXCITATION LIGHT SOURCE
SIGNAL BEAM
EXCITATION LIGHT
23-4
TRANSMISSION FIBER

FIG.5

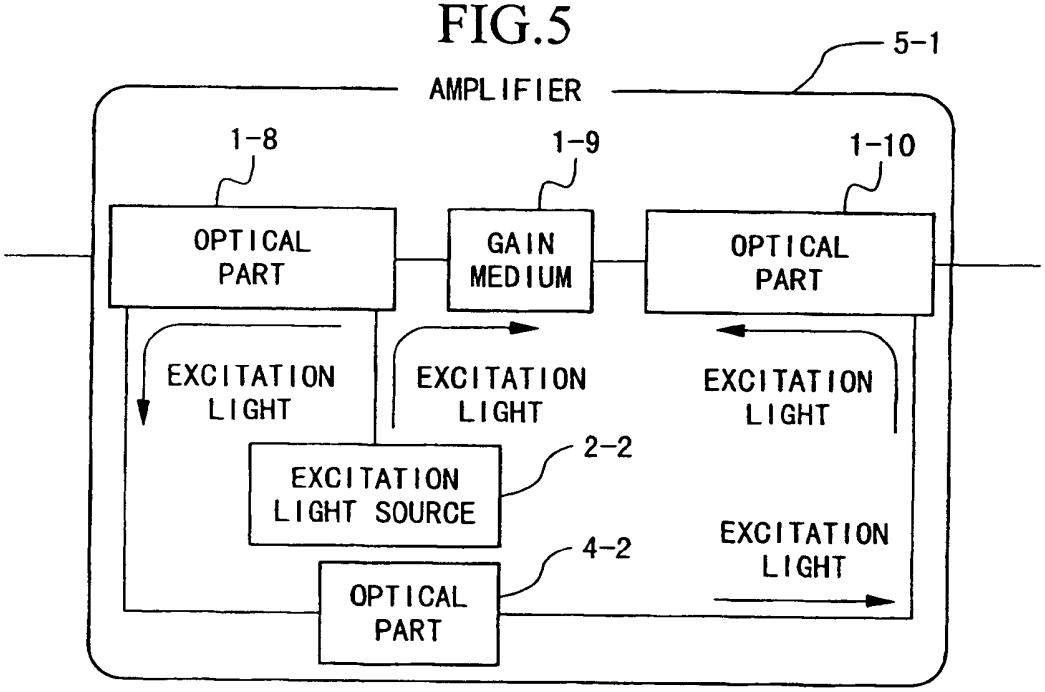

5-1
AMPLIFIER
1-8
1-9
1-10
OPTICAL PART
GAIN MEDIUM
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
2-2
EXCITATION LIGHT SOURCE
4-2
EXCITATION LIGHT
OPTICAL PART

FIG.6

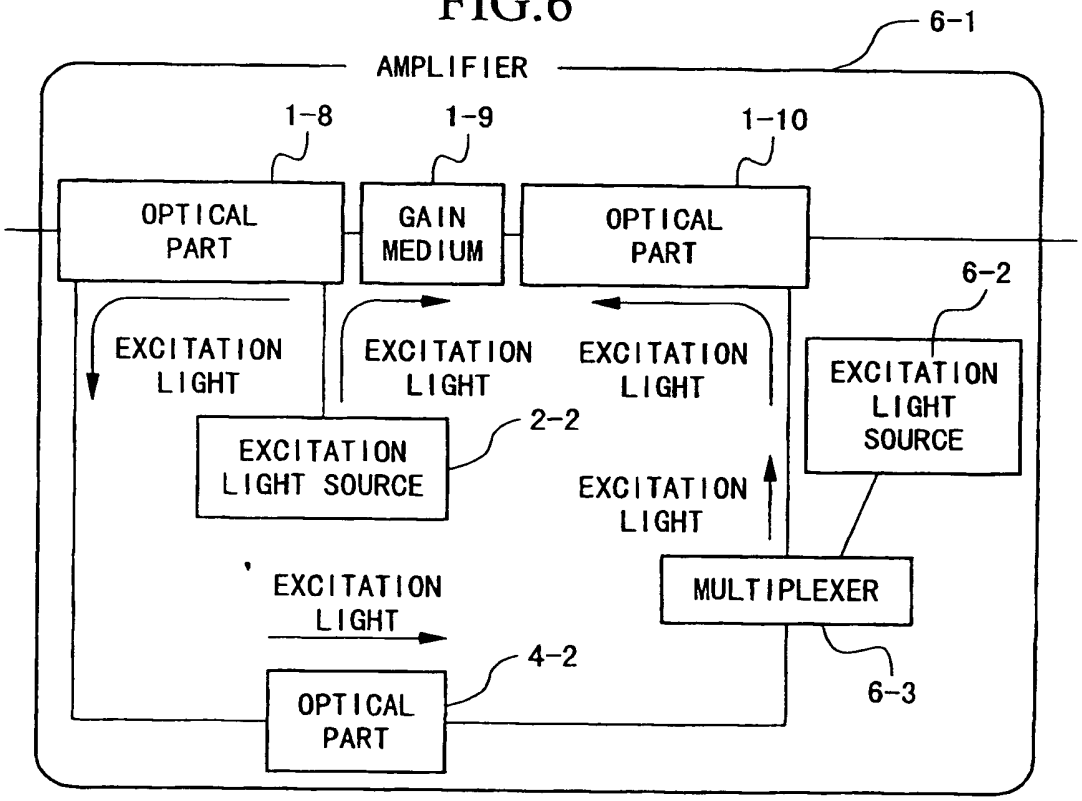

6-1
AMPLIFIER
1-8
1-9
1-10
OPTICAL PART
GAIN MEDIUM
OPTICAL PART
6-2
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
2-2
EXCITATION LIGHT SOURCE
EXCITATION LIGHT
EXCITATION LIGHT
MULTIPLEXER
6-3
4-2
OPTICAL PART

FIG.7A

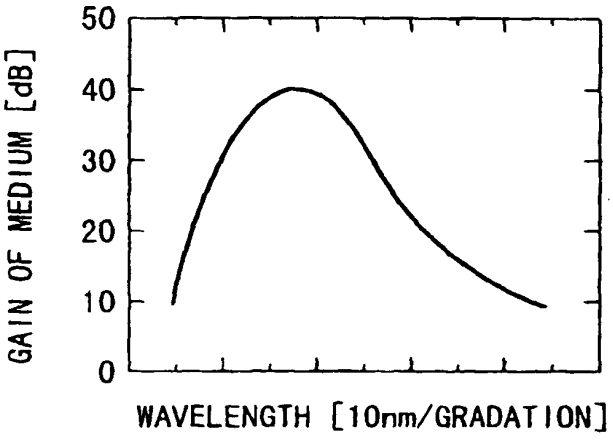


FIG.7B

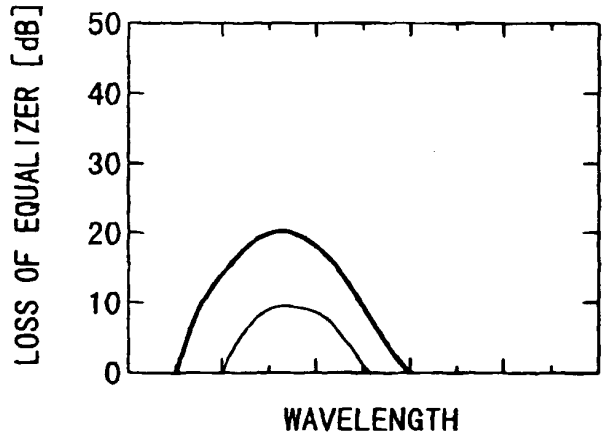


FIG.8

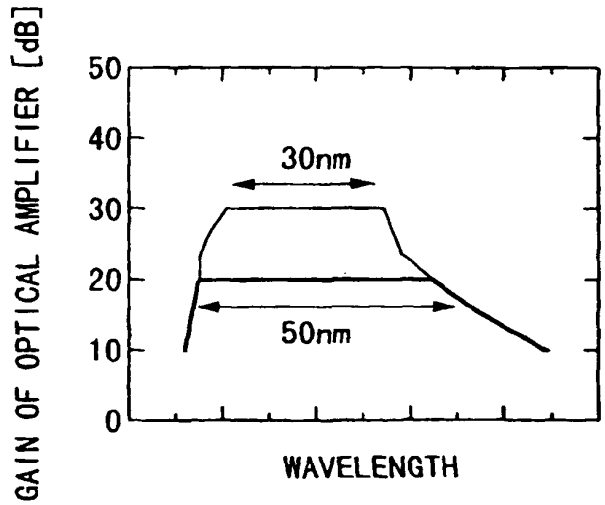


FIG.9A

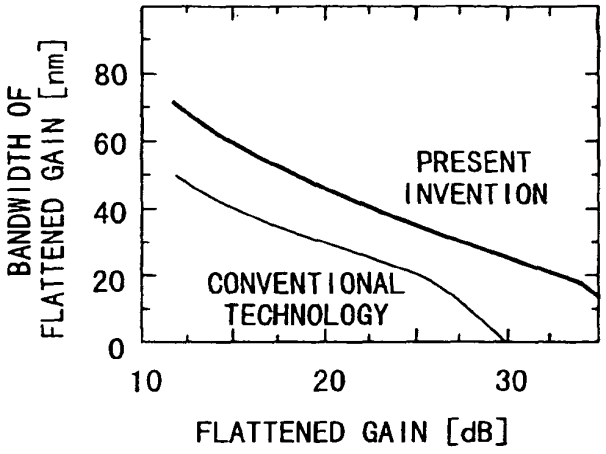


FIG.9B

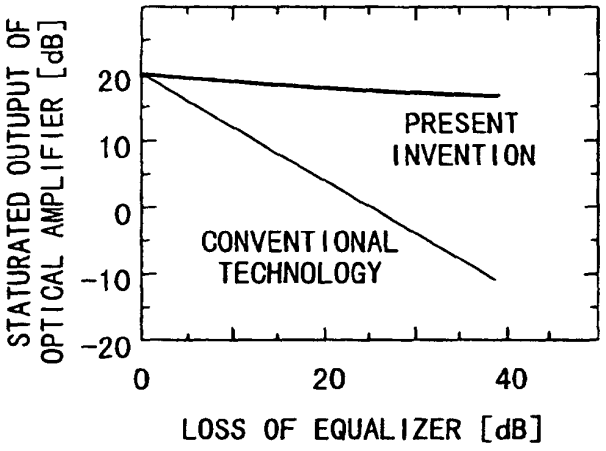


FIG.10

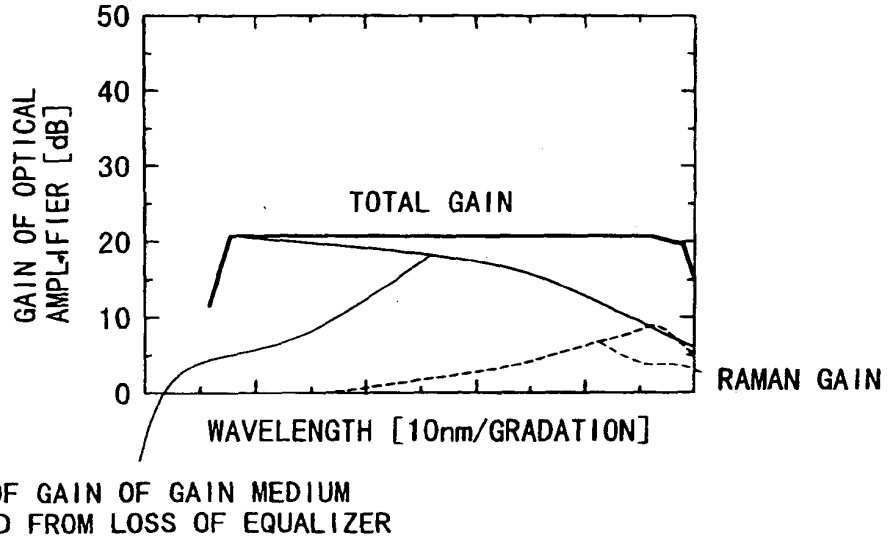


FIG.11A

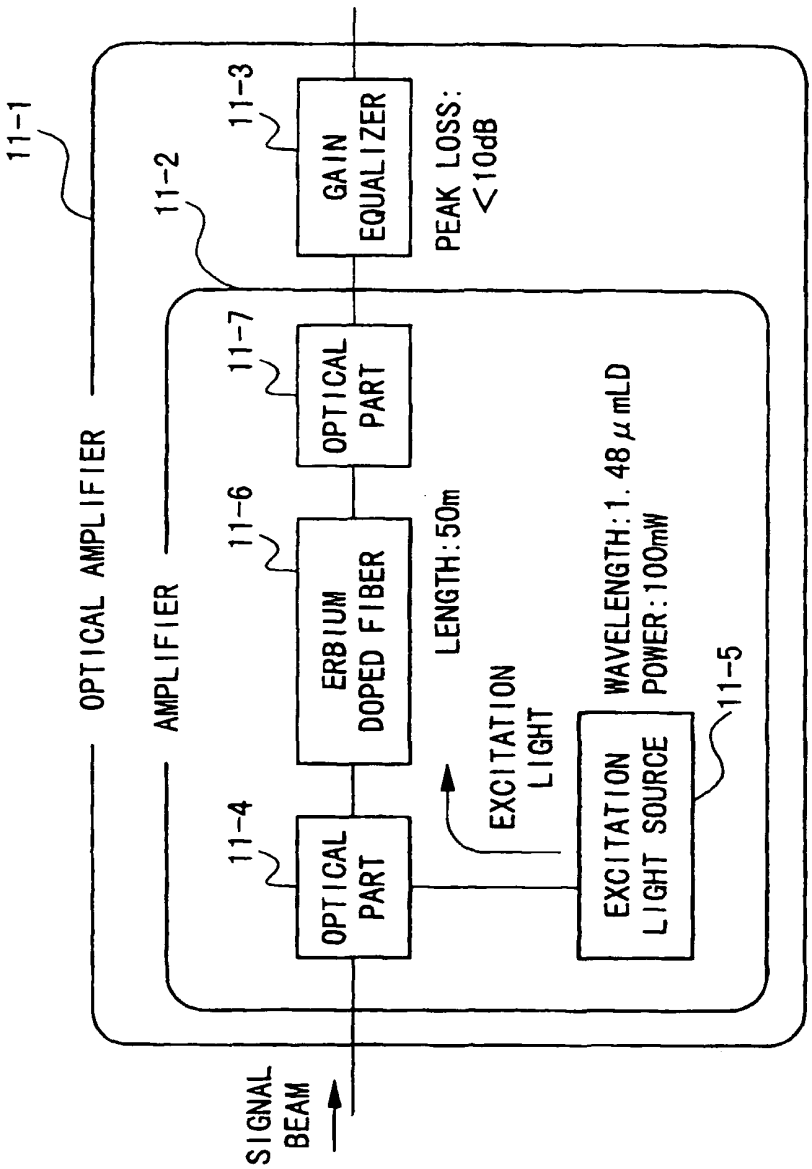

11-1
OPTICAL AMPLIFIER
11-2
AMPLIFIER
11-3
GAIN EQUALIZER
PEAK LOSS: <10dB
11-7
OPTICAL PART
11-6
ERBIUM DOPED FIBER
LENGTH:50m
11-4
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
WAVELENGTH:1.48μmLD
POWER:100mW
11-5
SIGNAL BEAM

FIG.11B

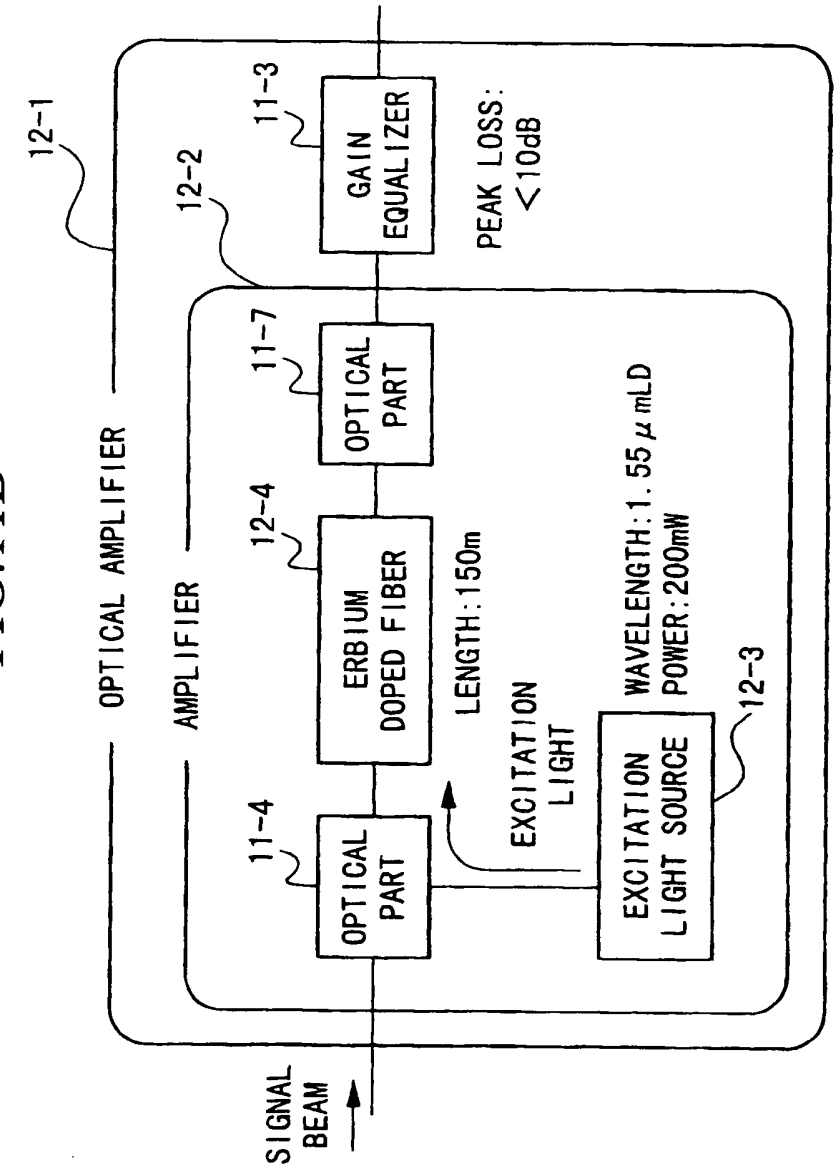

12-1
OPTICAL AMPLIFIER
12-2
AMPLIFIER
11-3
GAIN EQUALIZER
PEAK LOSS: <10dB
11-7
OPTICAL PART
12-4
ERBIUM DOPED FIBER
LENGTH:150m
11-4
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
WAVELENGTH:1.55μmLD
POWER:200mW
12-3
SIGNAL BEAM

FIG.12A

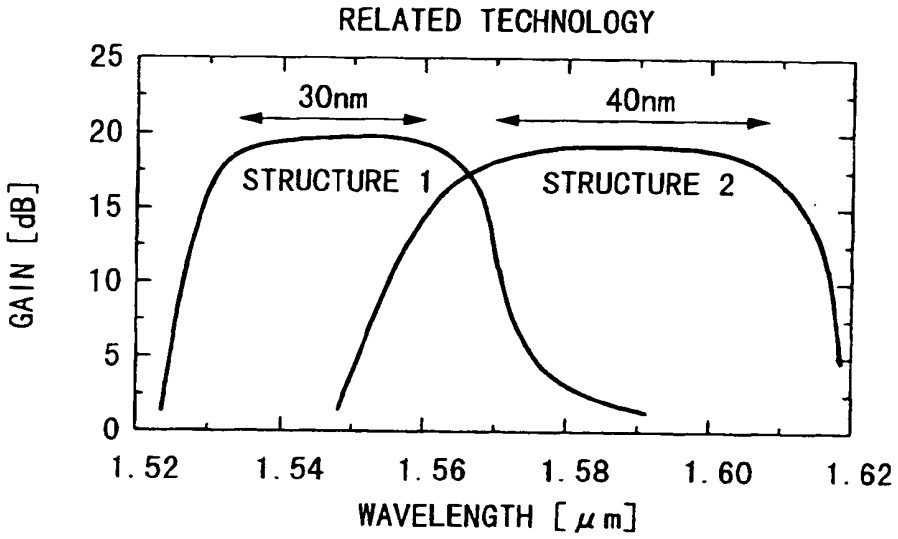


FIG.12B

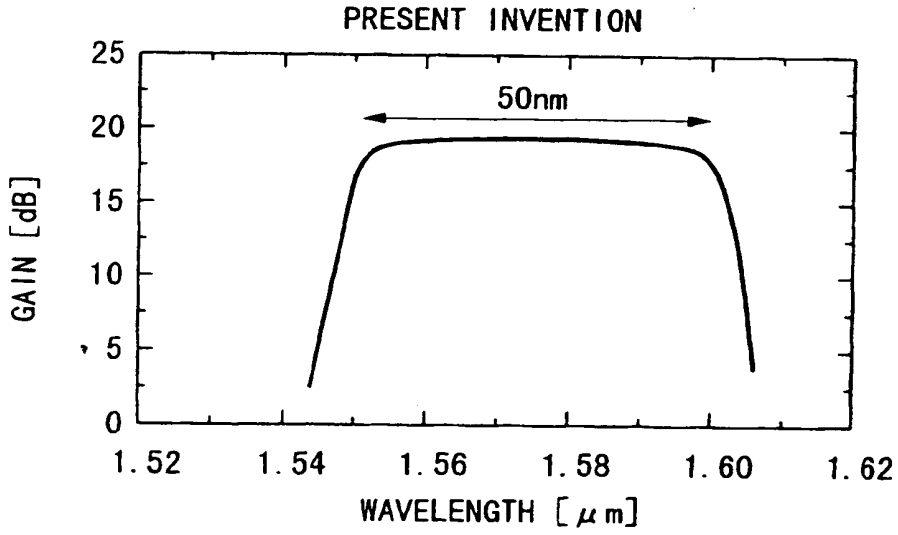


FIG.13

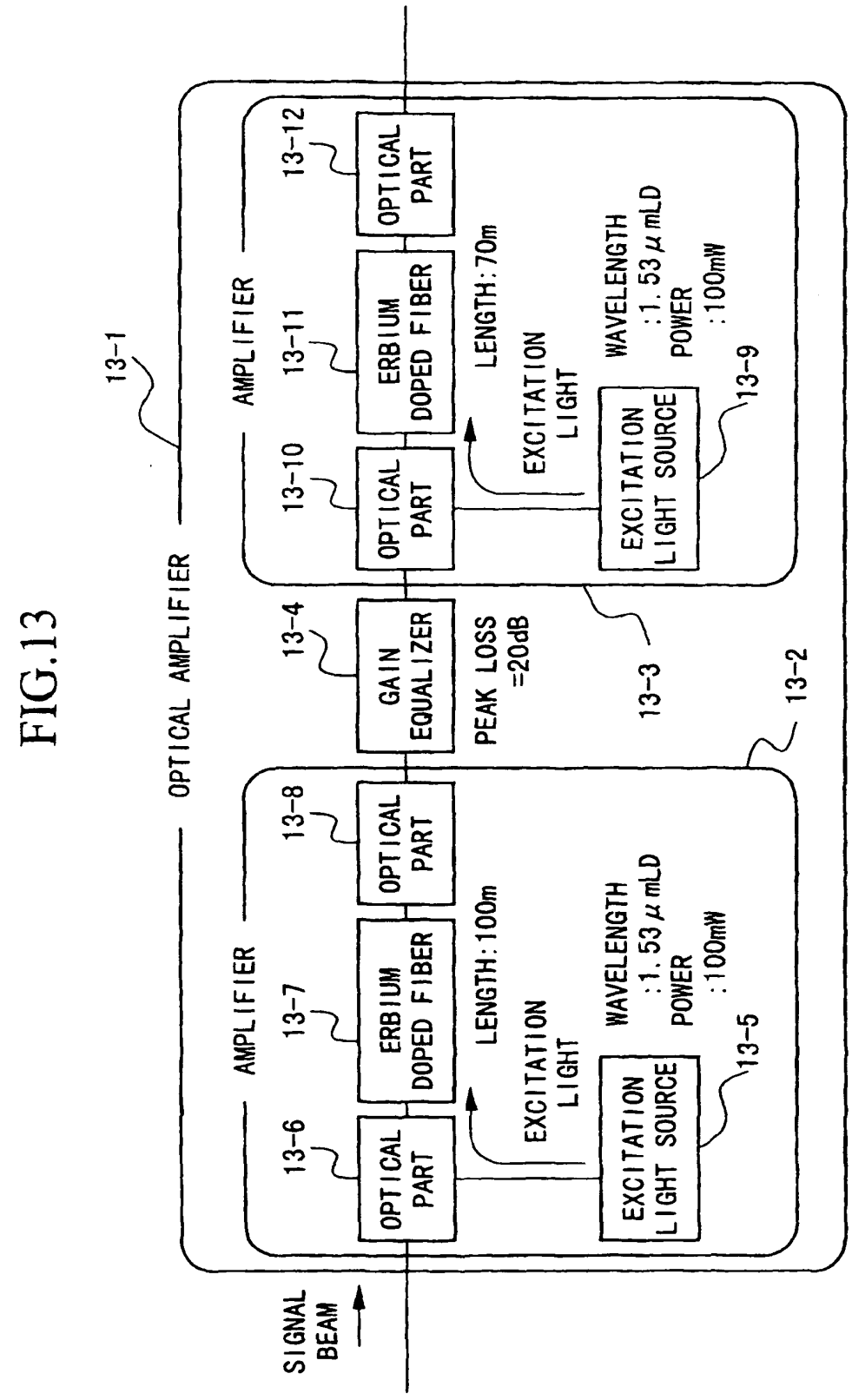

13-1
OPTICAL AMPLIFIER
AMPLIFIER
13-6
OPTICAL PART
13-7
ERBIUM DOPED FIBER
13-8
OPTICAL PART
LENGTH: 100m
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
WAVELENGTH :1.53 μ mLD
POWER :100mW
13-5
13-4
GAIN EQUALIZER
PEAK LOSS =20dB
13-3
13-2
AMPLIFIER
13-10
OPTICAL PART
13-11
ERBIUM DOPED FIBER
13-12
OPTICAL PART
LENGTH: 70m
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
WAVELENGTH :1.53 μ mLD
POWER :100mW
13-9
SIGNAL BEAM

FIG.14

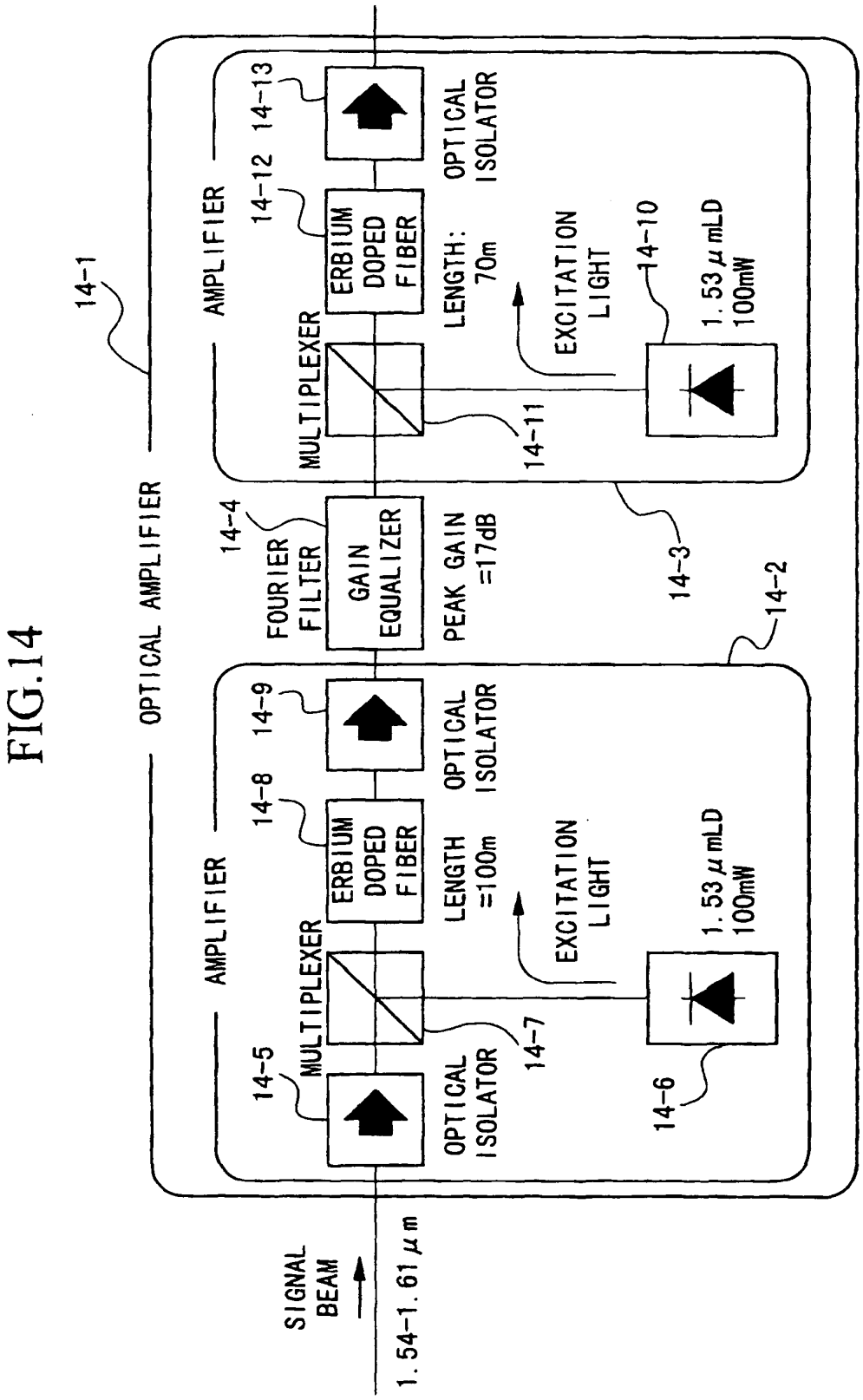

14-1
OPTICAL AMPLIFIER
AMPLIFIER
AMPLIFIER
14-2
14-3
14-4
FOURIER FILTER
GAIN EQUALIZER
PEAK GAIN =17dB
14-5
MULTIPLEXER
14-6
1.53μmLD 100mW
14-7
OPTICAL ISOLATOR
EXCITATION LIGHT
LENGTH =100m
14-8
ERBIUM DOPED FIBER
14-9
OPTICAL ISOLATOR
14-10
1.53μmLD 100mW
14-11
MULTIPLEXER
EXCITATION LIGHT
LENGTH: 70m
14-12
ERBIUM DOPED FIBER
14-13
OPTICAL ISOLATOR
SIGNAL BEAM
1.54-1.61μm

FIG.15

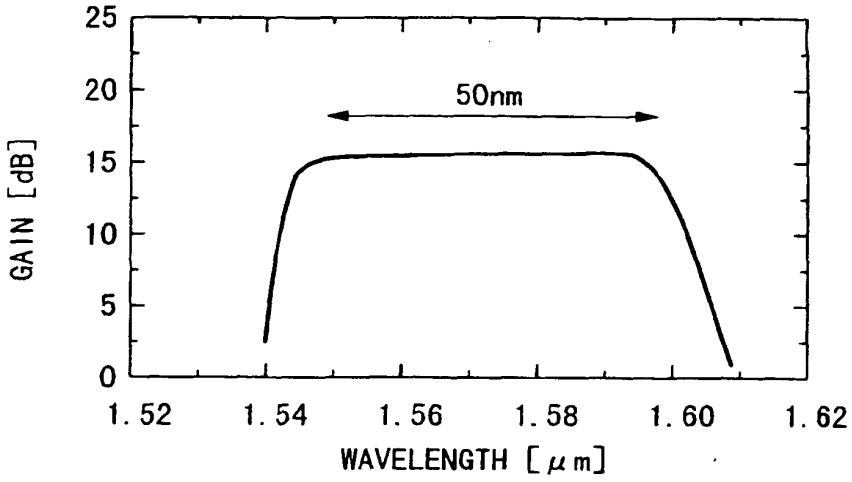


FIG.17

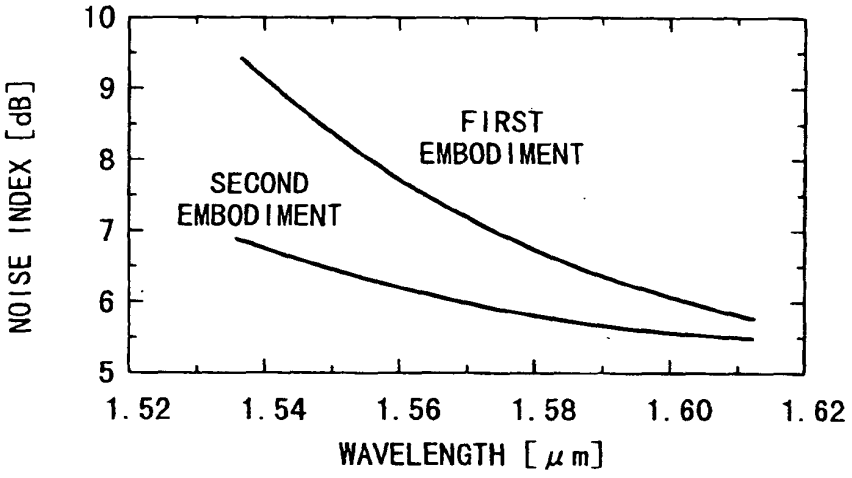


FIG.19

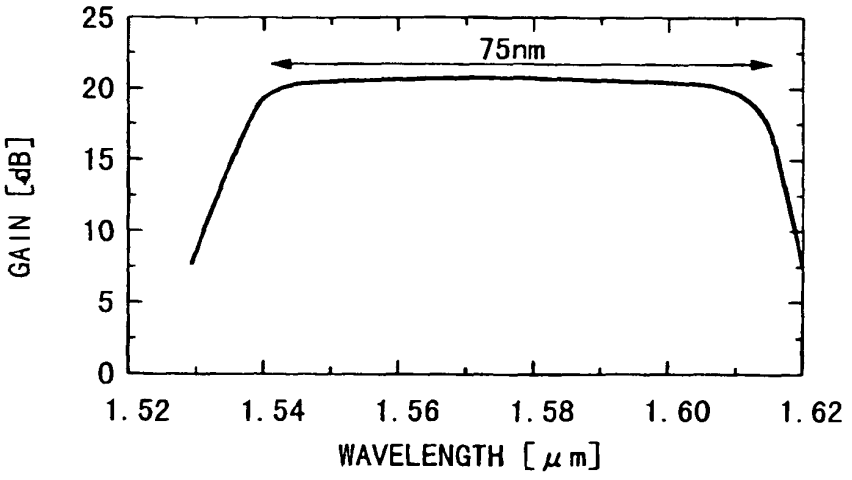


FIG.16

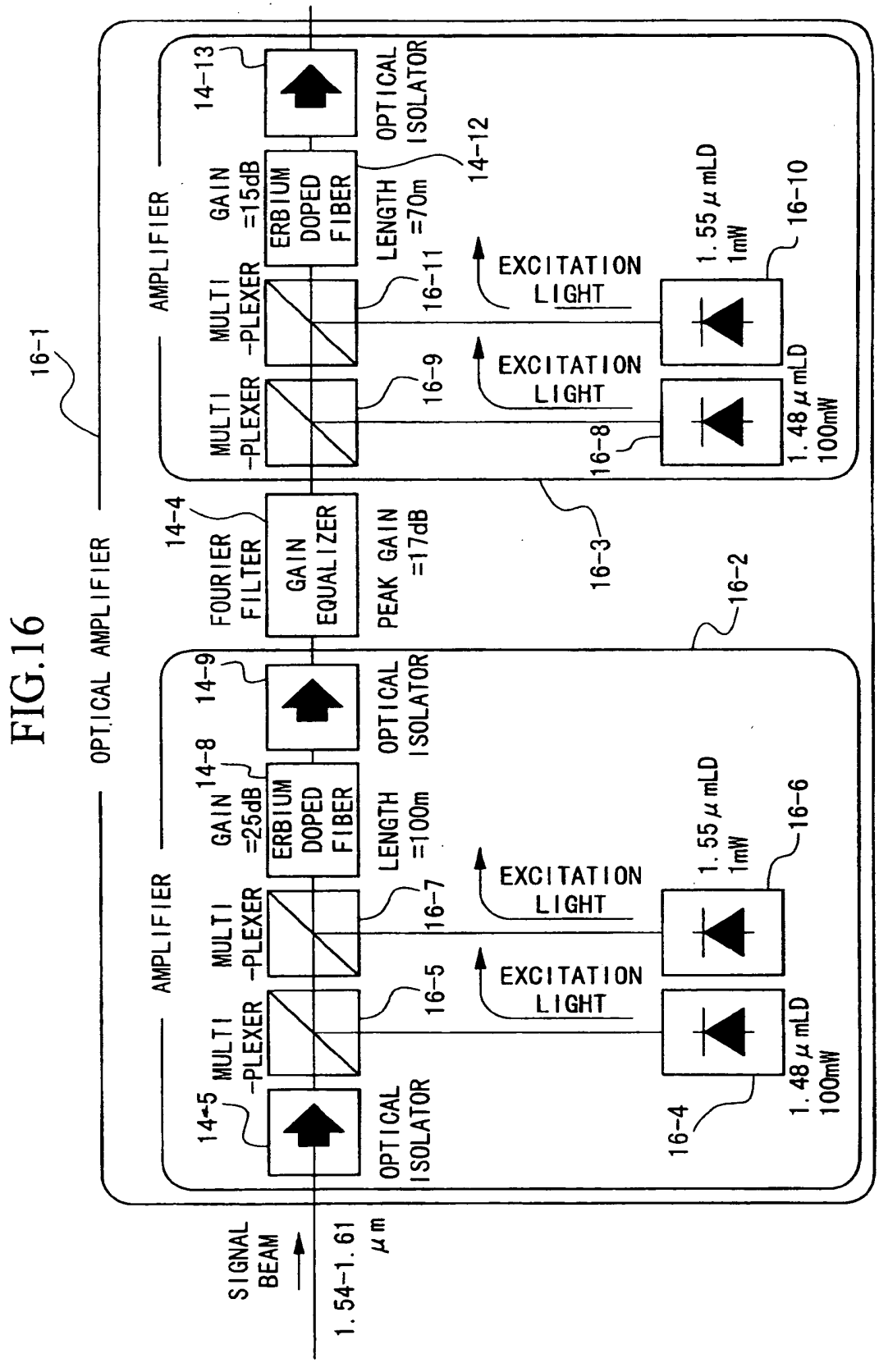

16-1
OPTICAL AMPLIFIER
AMPLIFIER
AMPLIFIER
SIGNAL BEAM
1.54-1.61 μm
14-5
OPTICAL ISOLATOR
MULTI -PLEXER
16-5
16-7
14-8
GAIN =25dB
ERBIUM DOPED FIBER
LENGTH =100m
14-9
OPTICAL ISOLATOR
14-4
FOURIER FILTER
GAIN EQUALIZER
PEAK GAIN =17dB
16-2
16-3
MULTI -PLEXER
16-8
16-9
16-11
14-12
GAIN =15dB
ERBIUM DOPED FIBER
LENGTH =70m
14-13
OPTICAL ISOLATOR
EXCITATION LIGHT
1.48 μ mLD 100mW
16-4
1.55 μ mLD 1mW
16-6
16-10

FIG.18

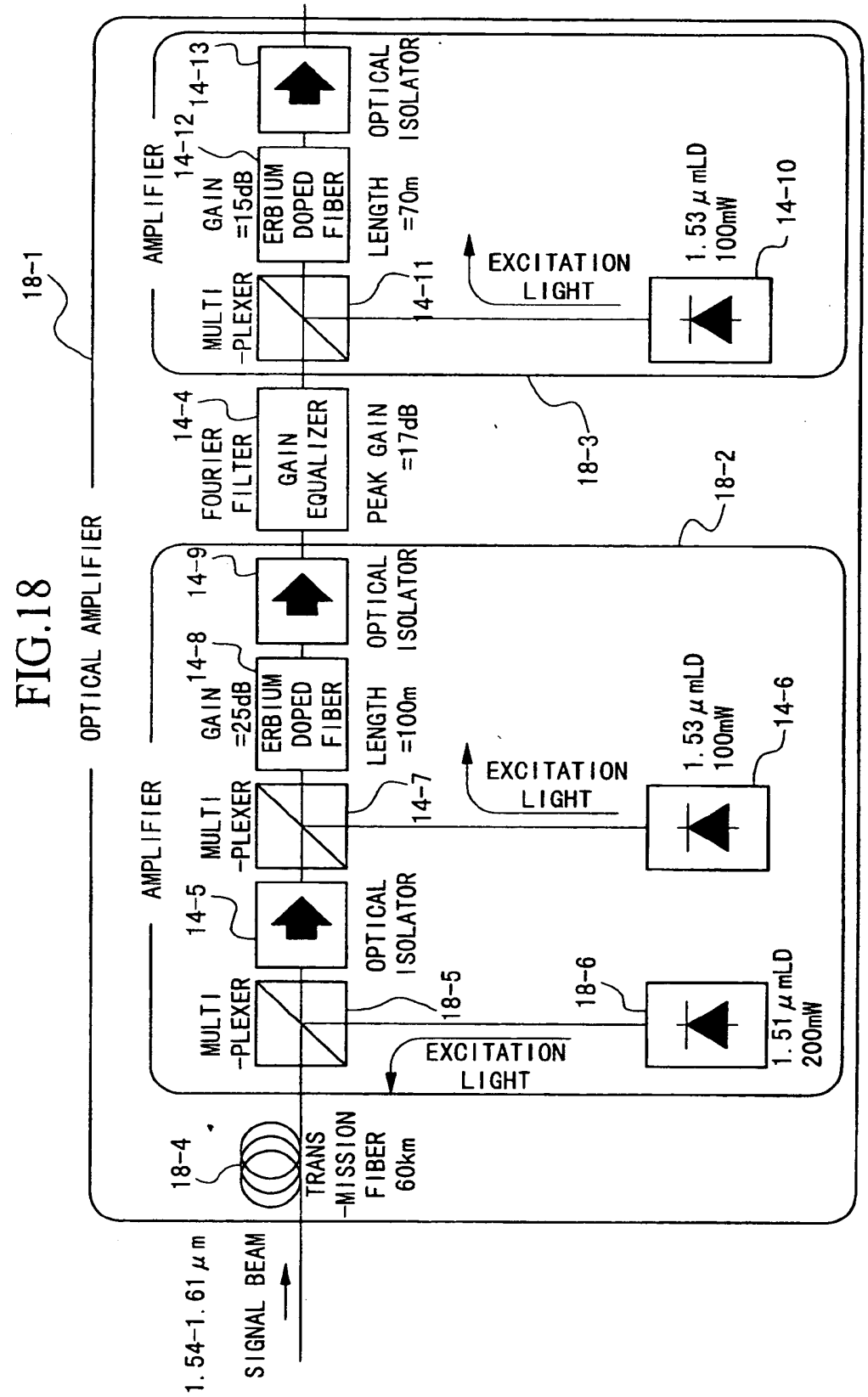

OPTICAL AMPLIFIER
18-1
AMPLIFIER
MULTI -PLEXER
14-5
OPTICAL ISOLATOR
GAIN =25dB
14-8
ERBIUM DOPED FIBER
14-9
OPTICAL ISOLATOR
LENGTH =100m
14-7
EXCITATION LIGHT
1.53 μmLD 100mW
14-6
18-5
18-6
EXCITATION LIGHT
1.51 μmLD 200mW
18-4
TRANS -MISSION FIBER 60km
1.54-1.61 μm
SIGNAL BEAM
14-4
FOURIER FILTER
GAIN EQUALIZER
PEAK GAIN =17dB
18-2
18-3
AMPLIFIER
MULTI -PLEXER
14-11
GAIN =15dB
14-12
ERBIUM DOPED FIBER
14-13
OPTICAL ISOLATOR
LENGTH =70m
EXCITATION LIGHT
1.53 μmLD 100mW
14-10

FIG.20

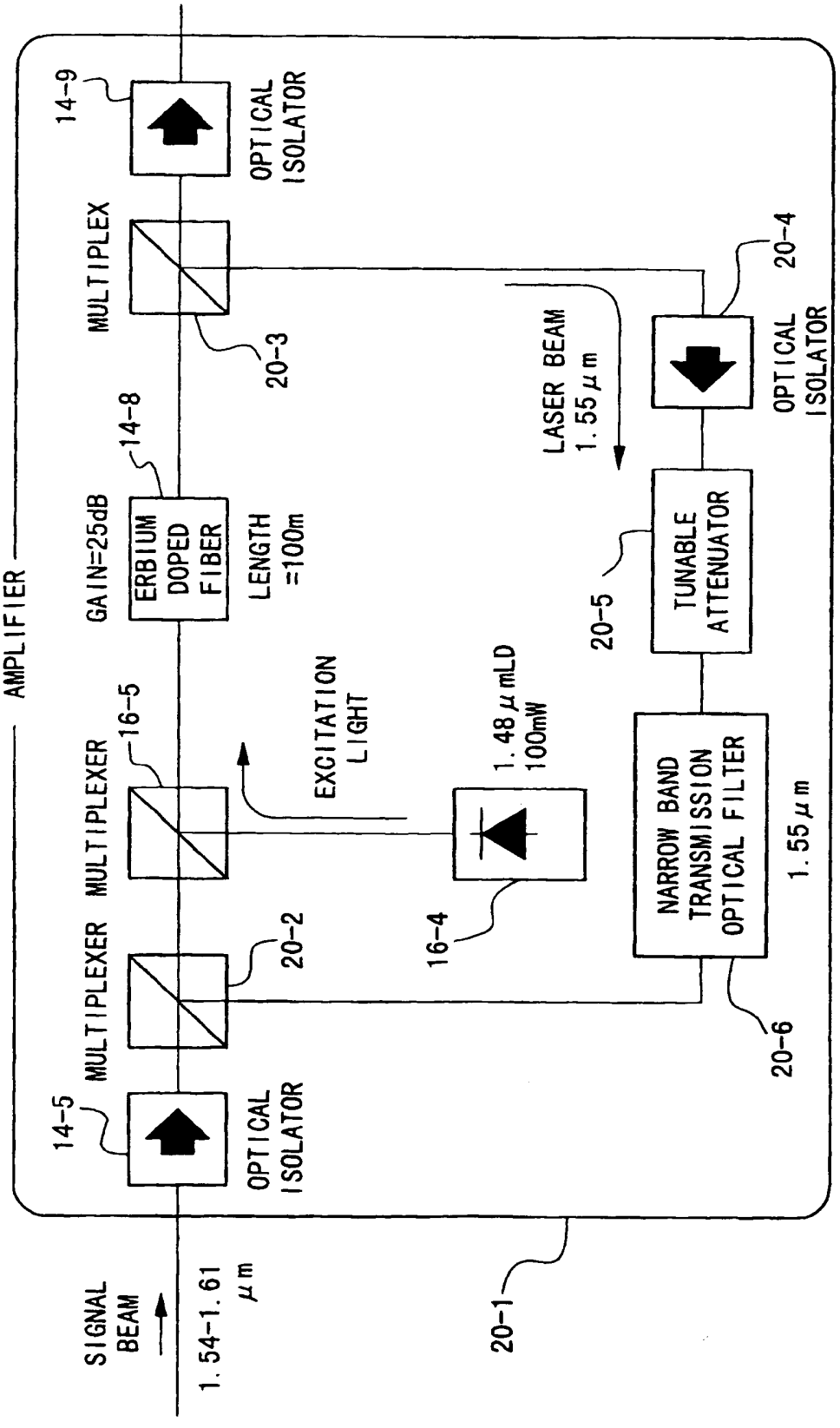

AMPLIFIER
SIGNAL BEAM
1.54-1.61 μm
14-5
OPTICAL ISOLATOR
MULTIPLEXER
20-2
16-5
MULTIPLEXER
EXCITATION LIGHT
16-4
1.48 μmLD 100mW
GAIN=25dB
14-8
ERBIUM DOPED FIBER
LENGTH =100m
MULTIPLEX
20-3
14-9
OPTICAL ISOLATOR
20-1
LASER BEAM 1.55 μm
20-5
TUNABLE ATTENUATOR
NARROW BAND TRANSMISSION OPTICAL FILTER
1.55 μm
20-6
20-4
OPTICAL ISOLATOR

FIG.21

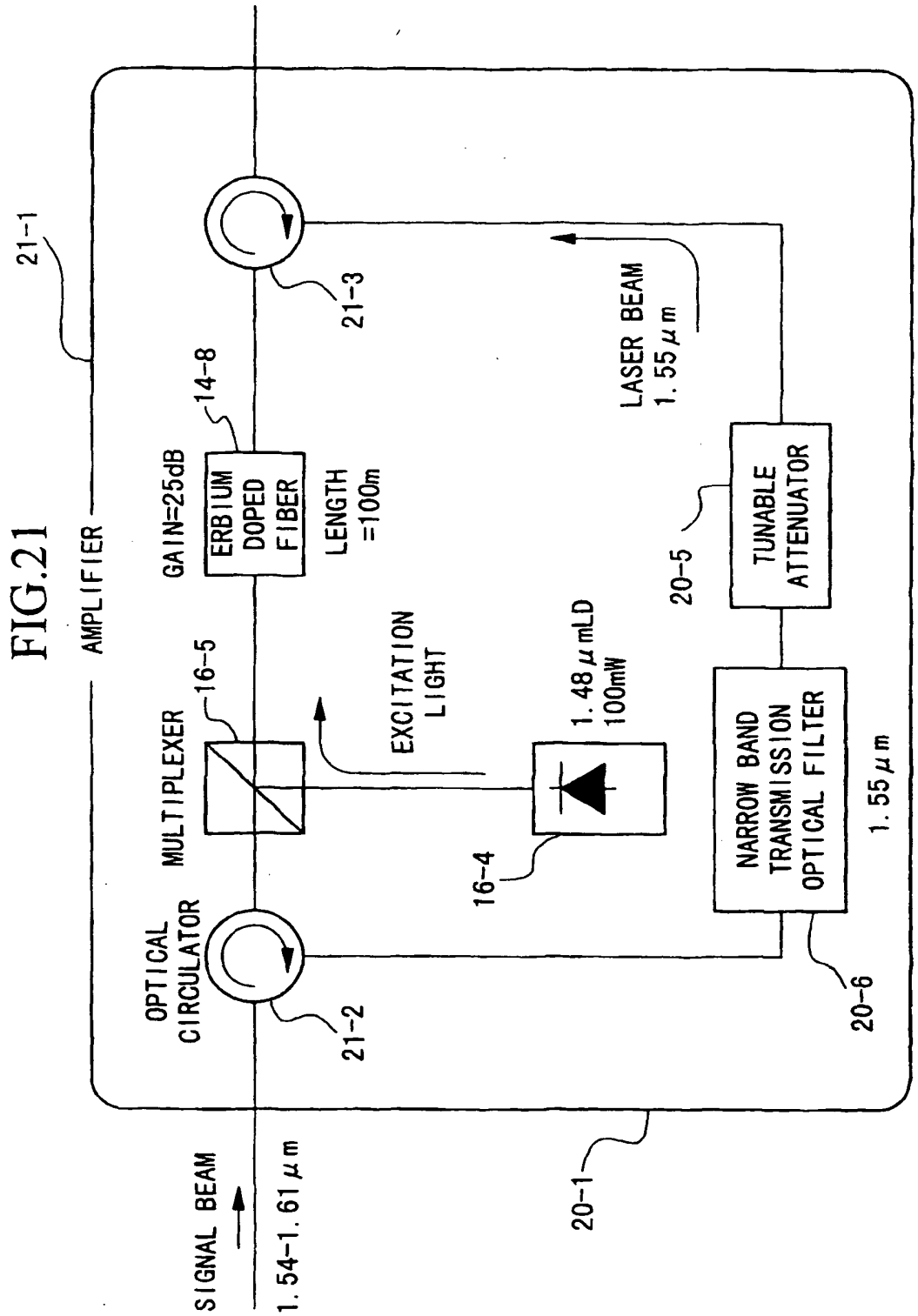

21-1
AMPLIFIER
SIGNAL BEAM
1.54-1.61μm
OPTICAL CIRCULATOR
21-2
MULTIPLEXER
16-5
EXCITATION LIGHT
1.48μmLD 100mW
16-4
GAIN=25dB
ERBIUM DOPED FIBER
14-8
LENGTH =100m
21-3
LASER BEAM 1.55μm
20-5
TUNABLE ATTENUATOR
NARROW BAND TRANSMISSION OPTICAL FILTER
1.55μm
20-6
20-1

FIG.22

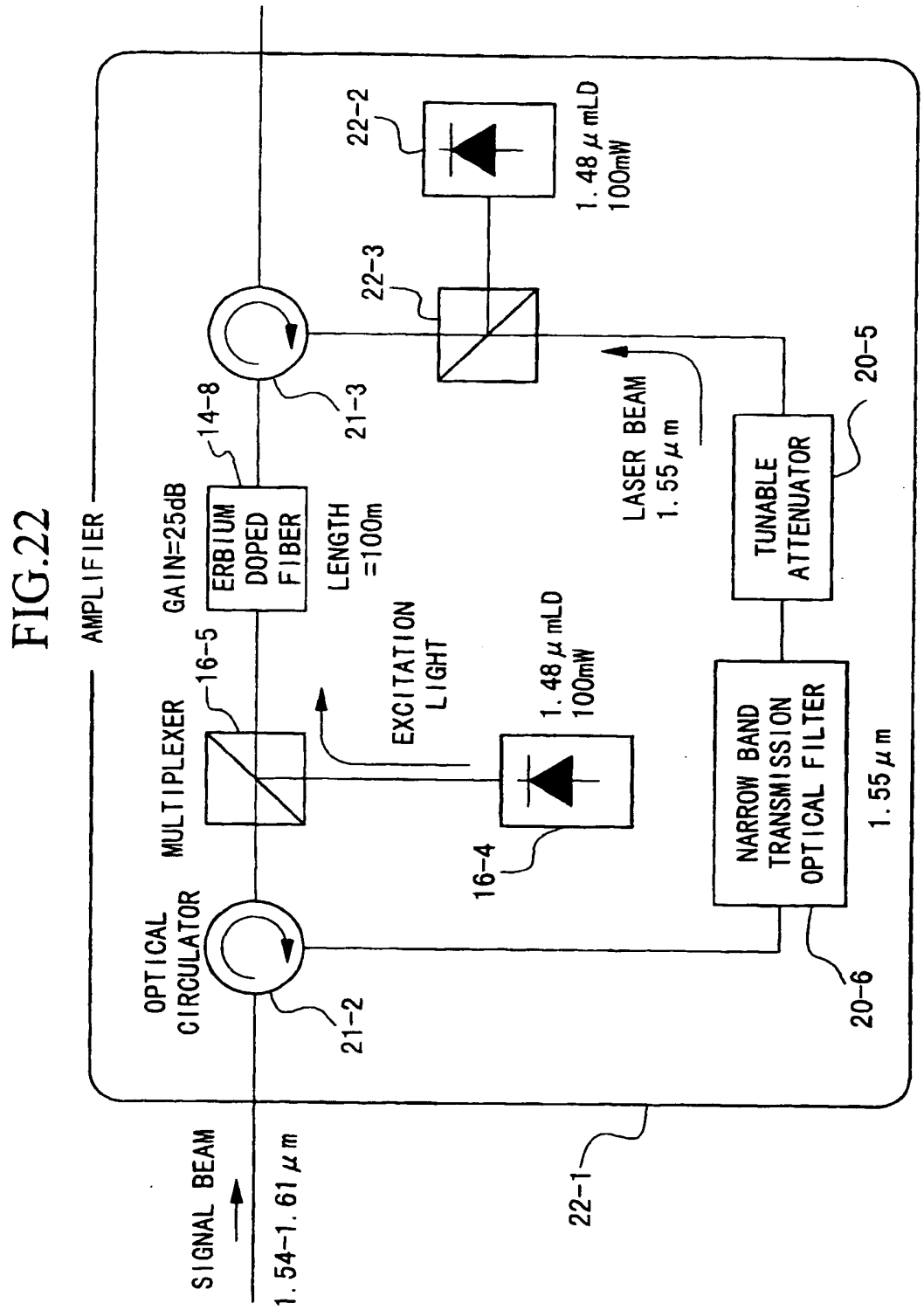

AMPLIFIER
SIGNAL BEAM
1.54-1.61 μm
OPTICAL CIRCULATOR
21-2
MULTIPLEXER
16-5
EXCITATION LIGHT
16-4
1.48 μmLD 100mW
GAIN=25dB
ERBIUM DOPED FIBER
14-8
LENGTH =100m
21-3
22-3
22-2
1.48 μmLD 100mW
LASER BEAM 1.55 μm
TUNABLE ATTENUATOR
20-5
NARROW BAND TRANSMISSION OPTICAL FILTER
1.55 μm
20-6
22-1

FIG.23

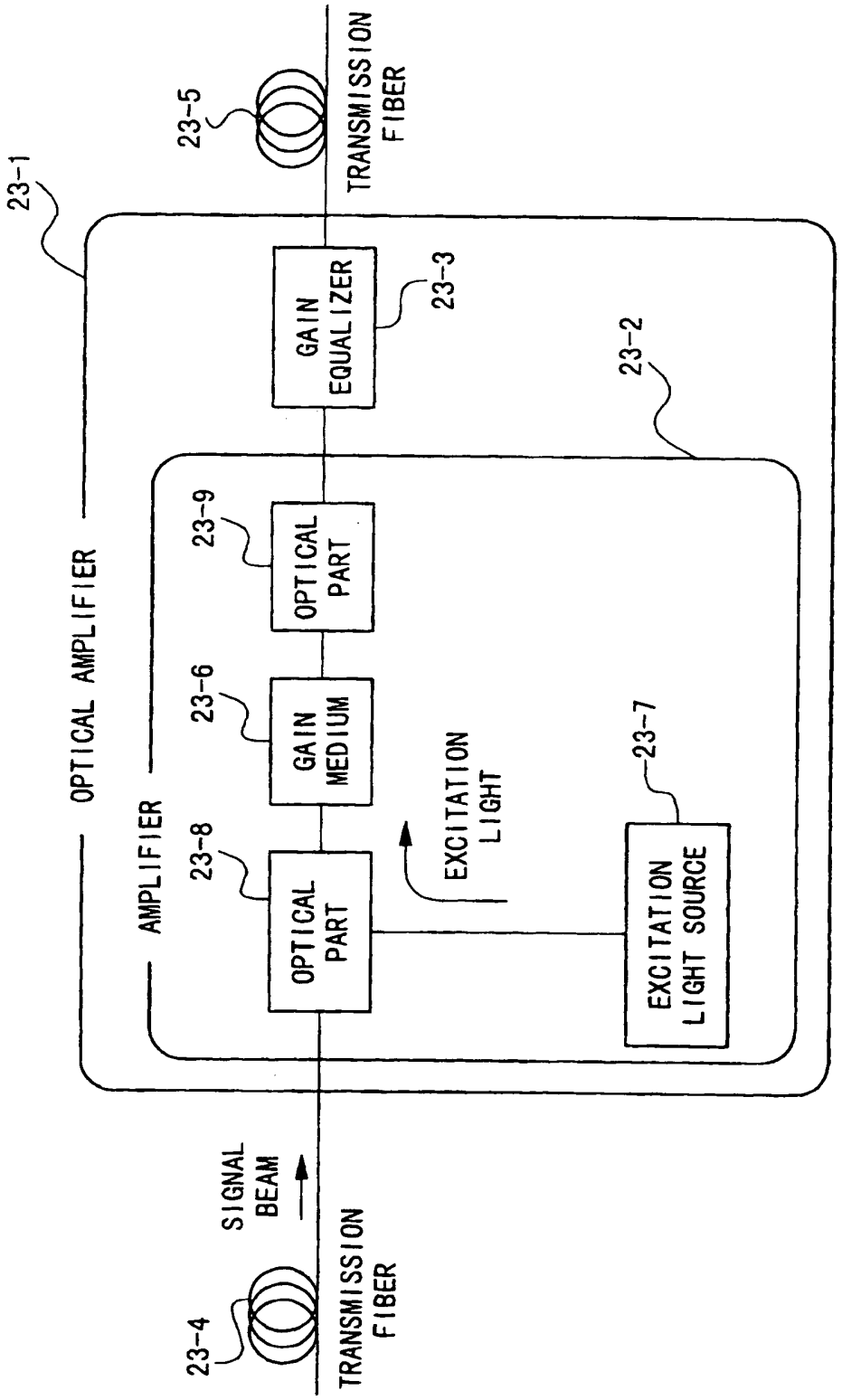

23-1
23-5
TRANSMISSION FIBER
GAIN EQUALIZER
23-3
23-2
23-9
OPTICAL PART
23-6
GAIN MEDIUM
OPTICAL AMPLIFIER
AMPLIFIER
23-8
OPTICAL PART
EXCITATION LIGHT
23-7
EXCITATION LIGHT SOURCE
SIGNAL BEAM
23-4
TRANSMISSION FIBER

FIG.24

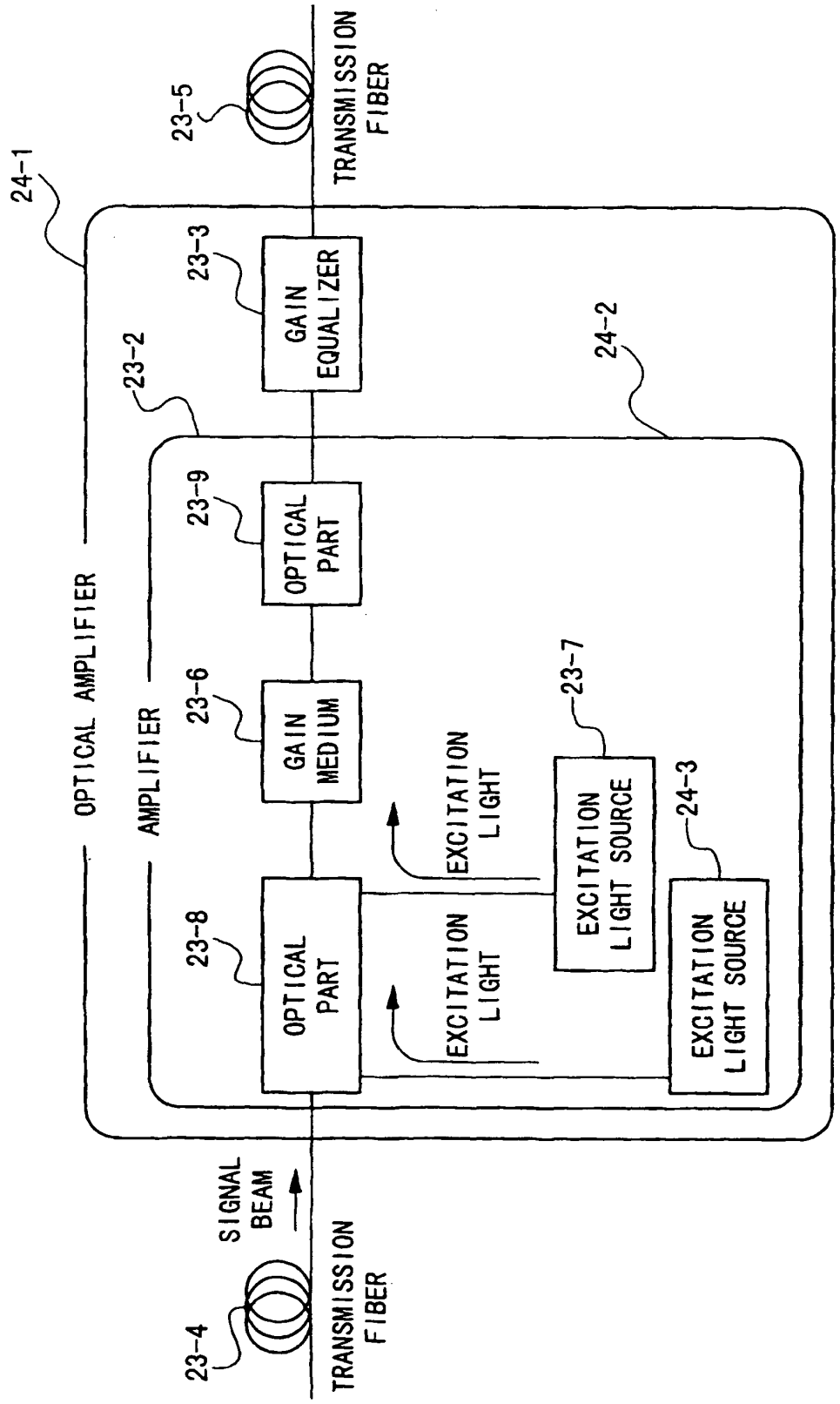

24-1
OPTICAL AMPLIFIER
23-5
TRANSMISSION FIBER
23-3
GAIN EQUALIZER
23-2
24-2
AMPLIFIER
23-9
OPTICAL PART
23-6
GAIN MEDIUM
EXCITATION LIGHT
23-7
EXCITATION LIGHT SOURCE
24-3
EXCITATION LIGHT SOURCE
23-8
OPTICAL PART
EXCITATION LIGHT
SIGNAL BEAM
23-4
TRANSMISSION FIBER

FIG.25

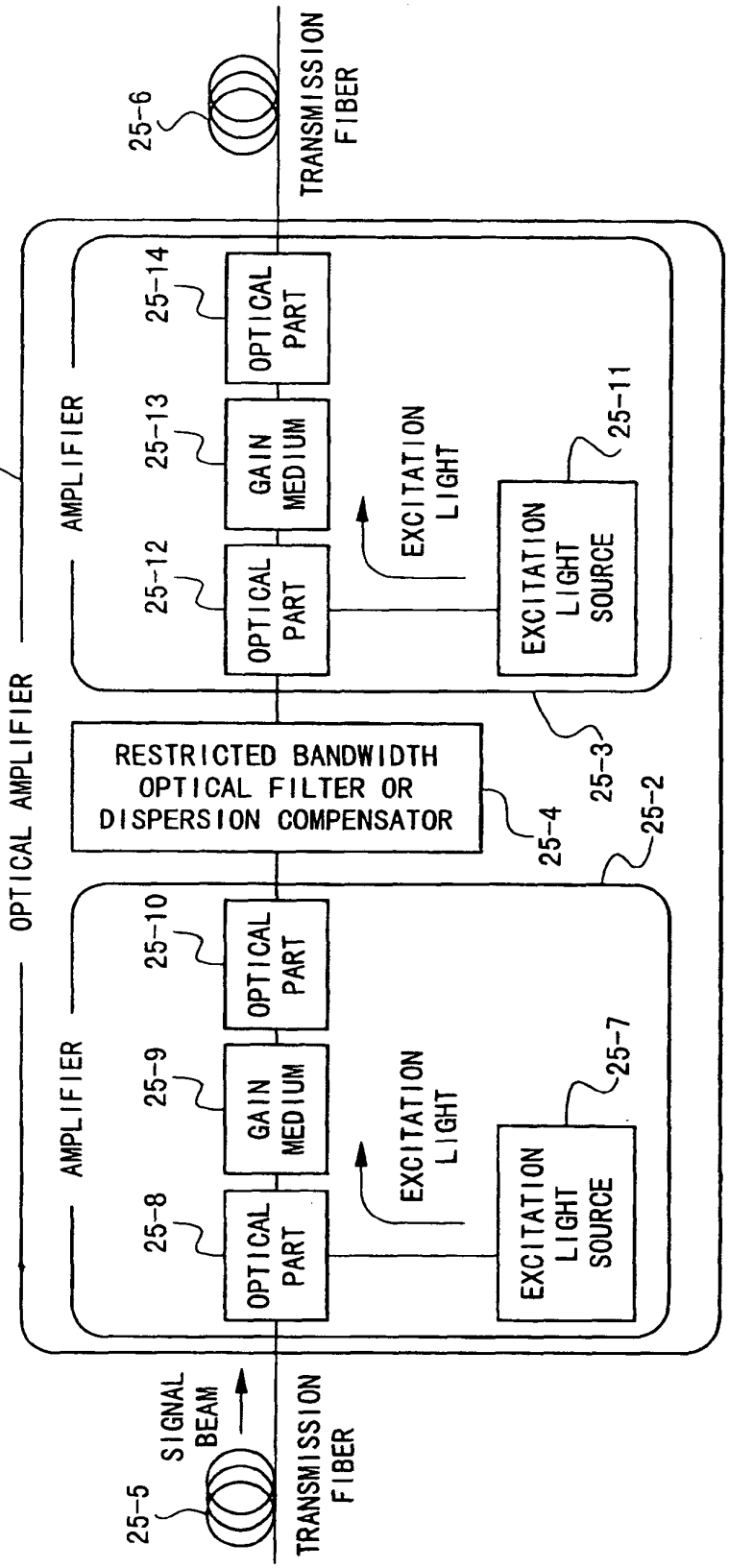

25-1
OPTICAL AMPLIFIER
AMPLIFIER
AMPLIFIER
25-5
SIGNAL BEAM
TRANSMISSION FIBER
25-6
TRANSMISSION FIBER
25-8
OPTICAL PART
25-9
GAIN MEDIUM
25-10
OPTICAL PART
EXCITATION LIGHT
25-7
EXCITATION LIGHT SOURCE
RESTRICTED BANDWIDTH OPTICAL FILTER OR DISPERSION COMPENSATOR
25-4
25-2
25-3
25-12
OPTICAL PART
25-13
GAIN MEDIUM
25-14
OPTICAL PART
EXCITATION LIGHT
25-11
EXCITATION LIGHT SOURCE

FIG.26A

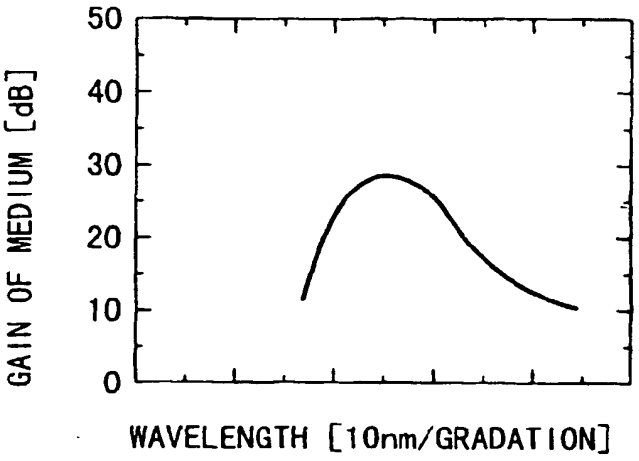


FIG.26B

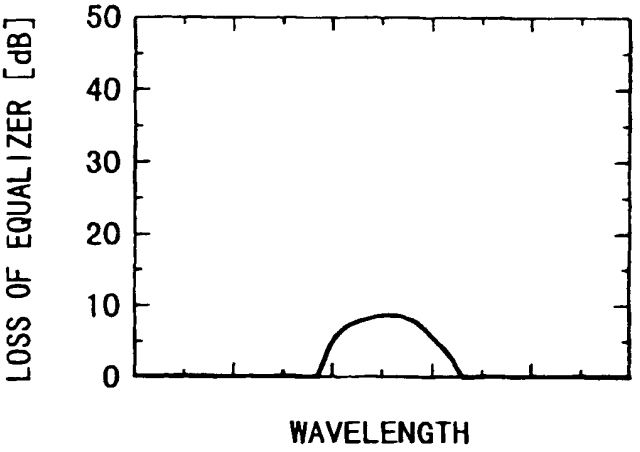


FIG.26C

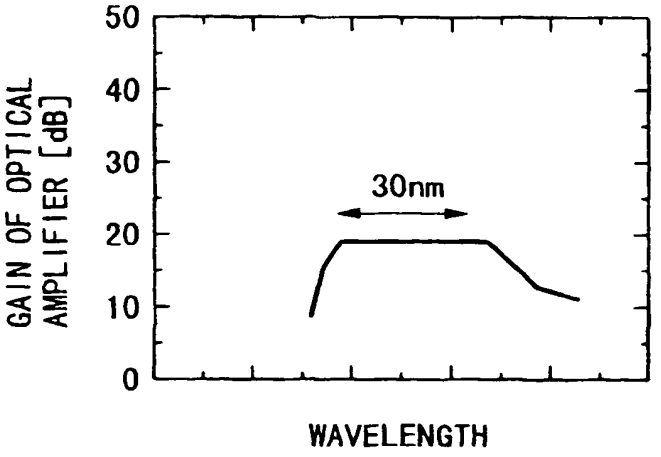


FIG.27

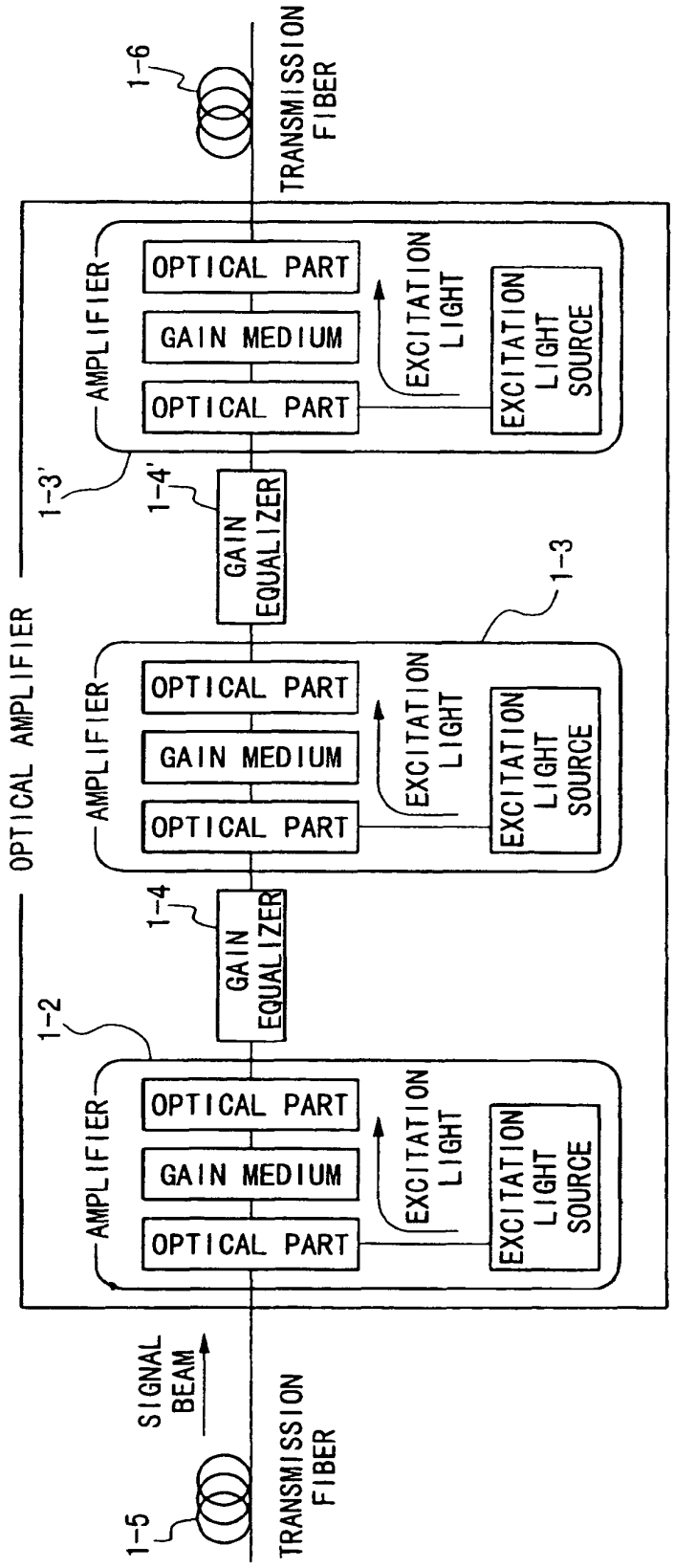
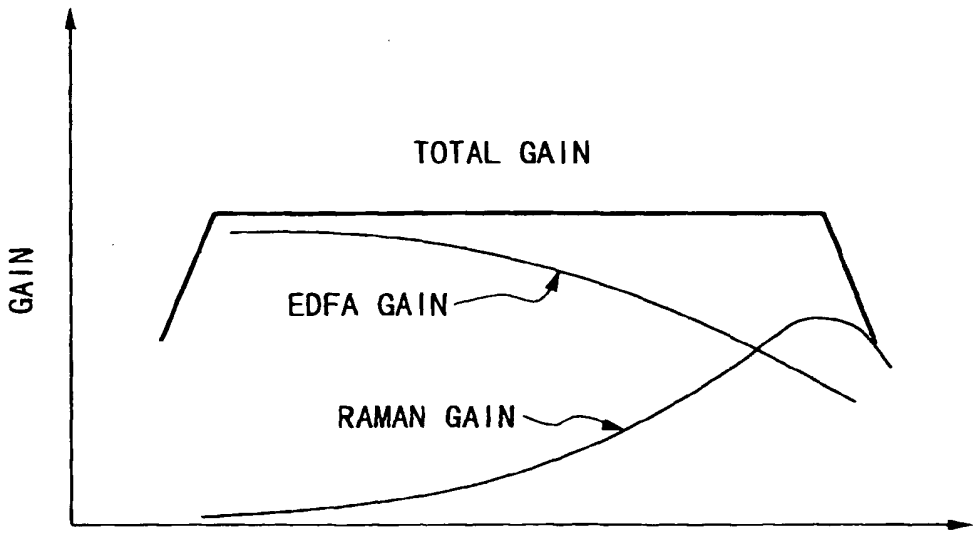

1-6
TRANSMISSION FIBER
OPTICAL AMPLIFIER
AMPLIFIER
OPTICAL PART
GAIN MEDIUM
OPTICAL PART
EXCITATION LIGHT
EXCITATION LIGHT SOURCE
1-3'
1-4'
GAIN EQUALIZER
1-3
1-4
1-2
SIGNAL BEAM
1-5
TRANSMISSION FIBER

FIG.28



GAIN SPECTRUM

FIG.29

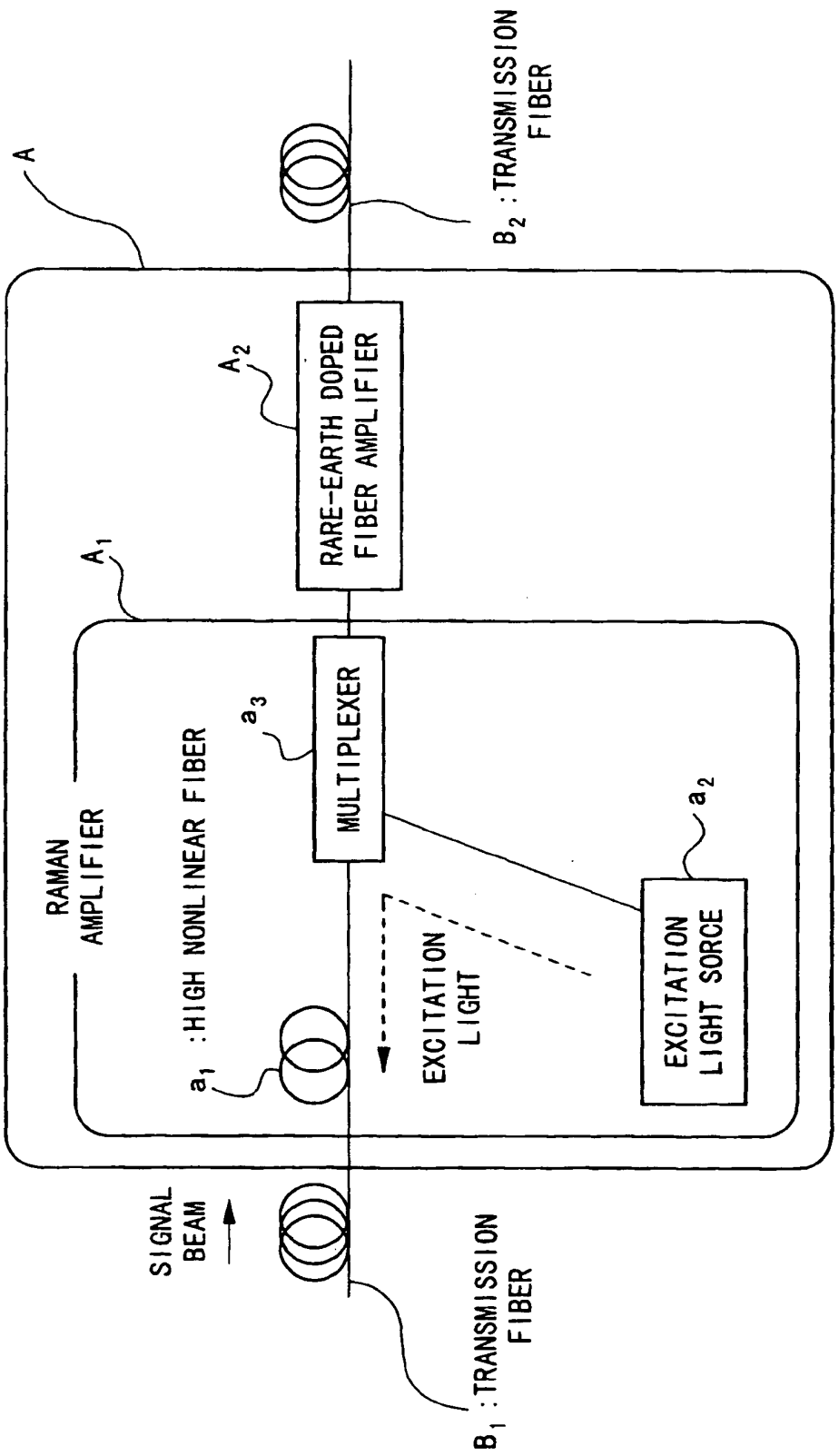

A
B_2 : TRANSMISSION FIBER
A_2
RARE-EARTH DOPED FIBER AMPLIFIER
A_1
RAMAN AMPLIFIER
a_3
MULTIPLEXER
a_1 : HIGH NONLINEAR FIBER
EXCITATION LIGHT
a_2
EXCITATION LIGHT SORCE
SIGNAL BEAM
B_1 : TRANSMISSION FIBER

FIG.30

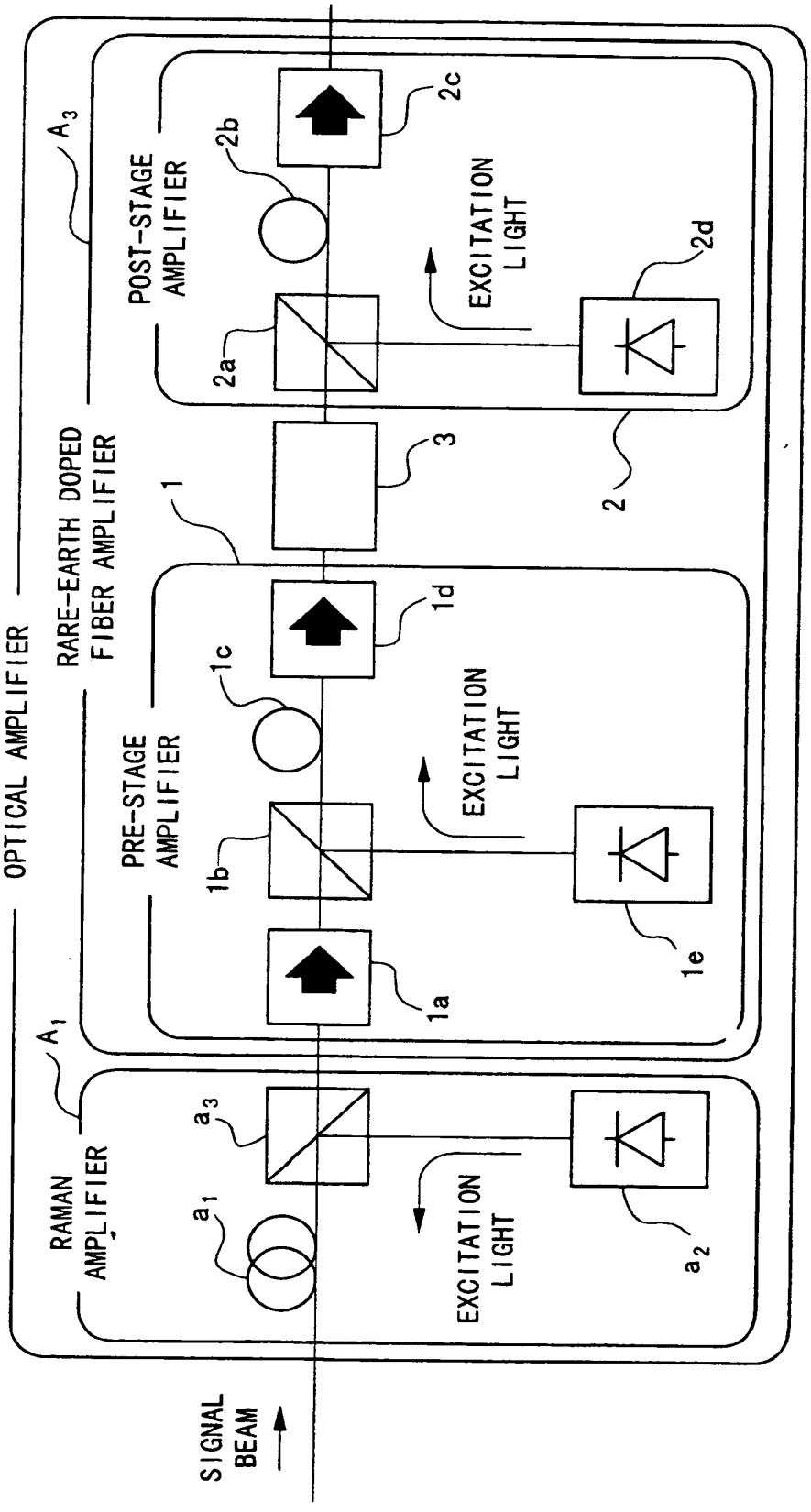

OPTICAL AMPLIFIER
A1
A3
RAMAN AMPLIFIER
RARE-EARTH DOPED FIBER AMPLIFIER
PRE-STAGE AMPLIFIER
POST-STAGE AMPLIFIER
SIGNAL BEAM
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
a1
a2
a3
1
1a
1b
1c
1d
1e
2
2a
2b
2c
2d
3

FIG.31

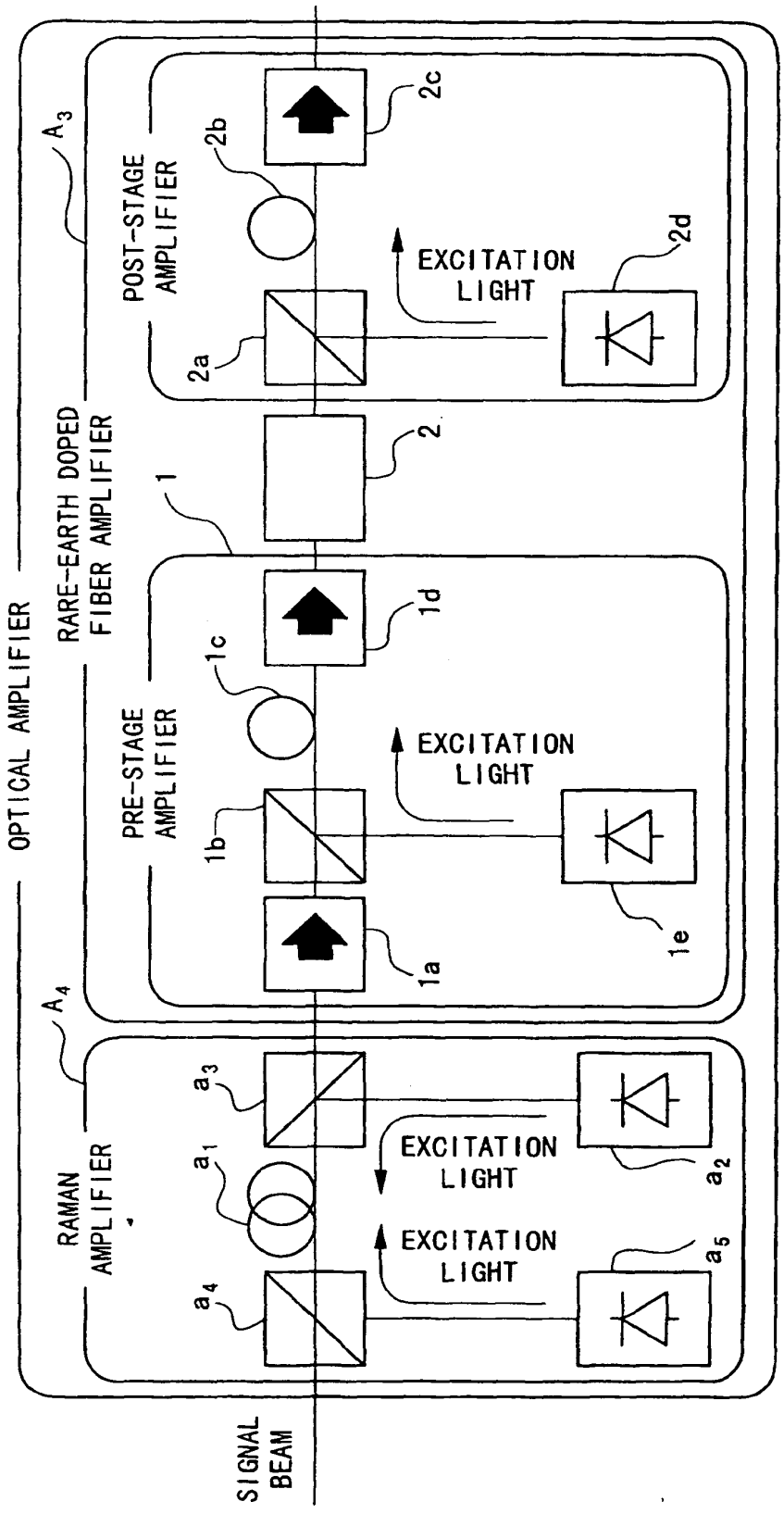

OPTICAL AMPLIFIER
A3
A4
RARE-EARTH DOPED FIBER AMPLIFIER
POST-STAGE AMPLIFIER
PRE-STAGE AMPLIFIER
RAMAN AMPLIFIER
SIGNAL BEAM
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
EXCITATION LIGHT
1
1a
1b
1c
1d
1e
2
2a
2b
2c
2d
a1
a2
a3
a4
a5

FIG.32

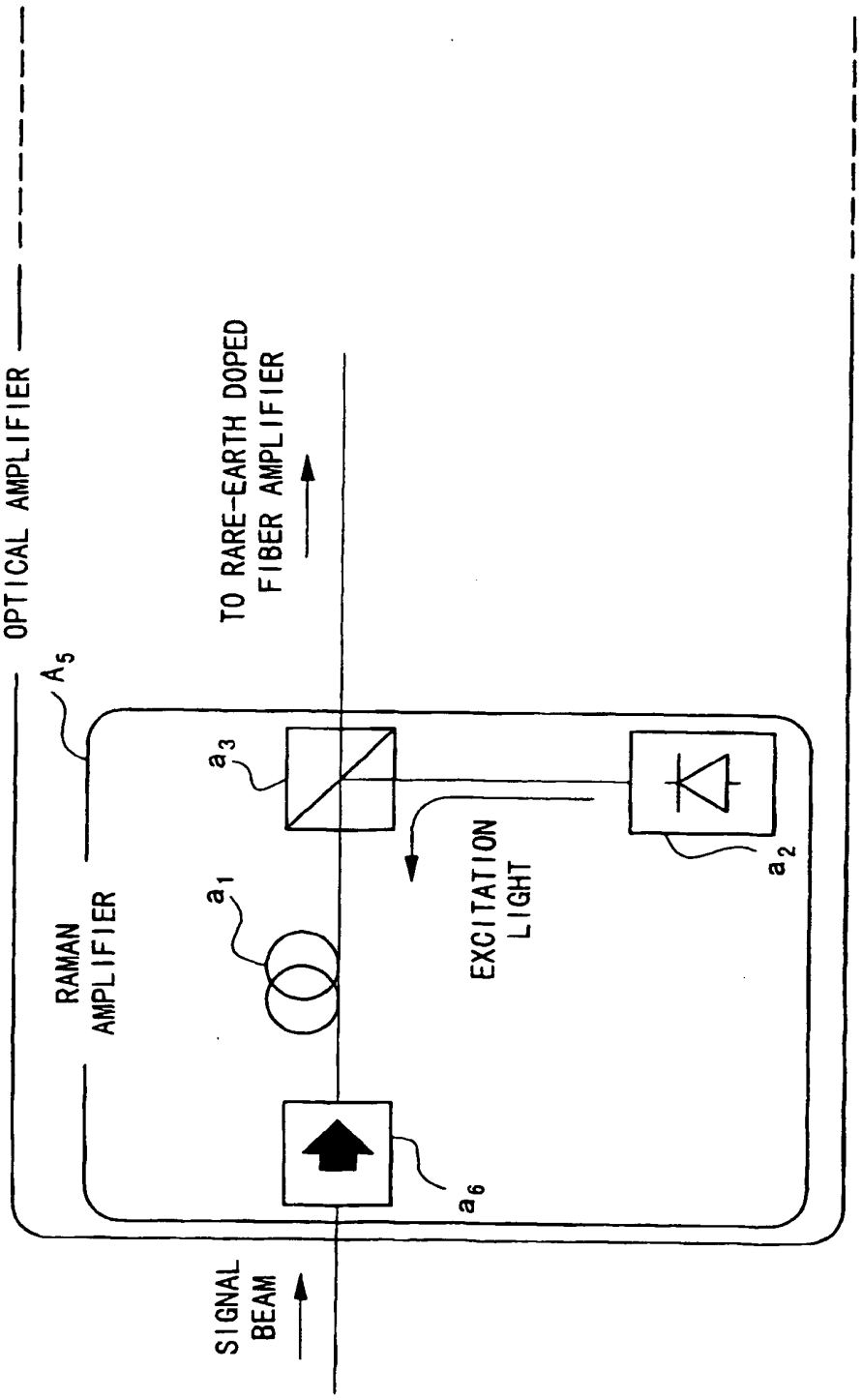

OPTICAL AMPLIFIER
A5
TO RARE-EARTH DOPED FIBER AMPLIFIER
a3
a1
RAMAN AMPLIFIER
EXCITATION LIGHT
a2
a6
SIGNAL BEAM

FIG.33

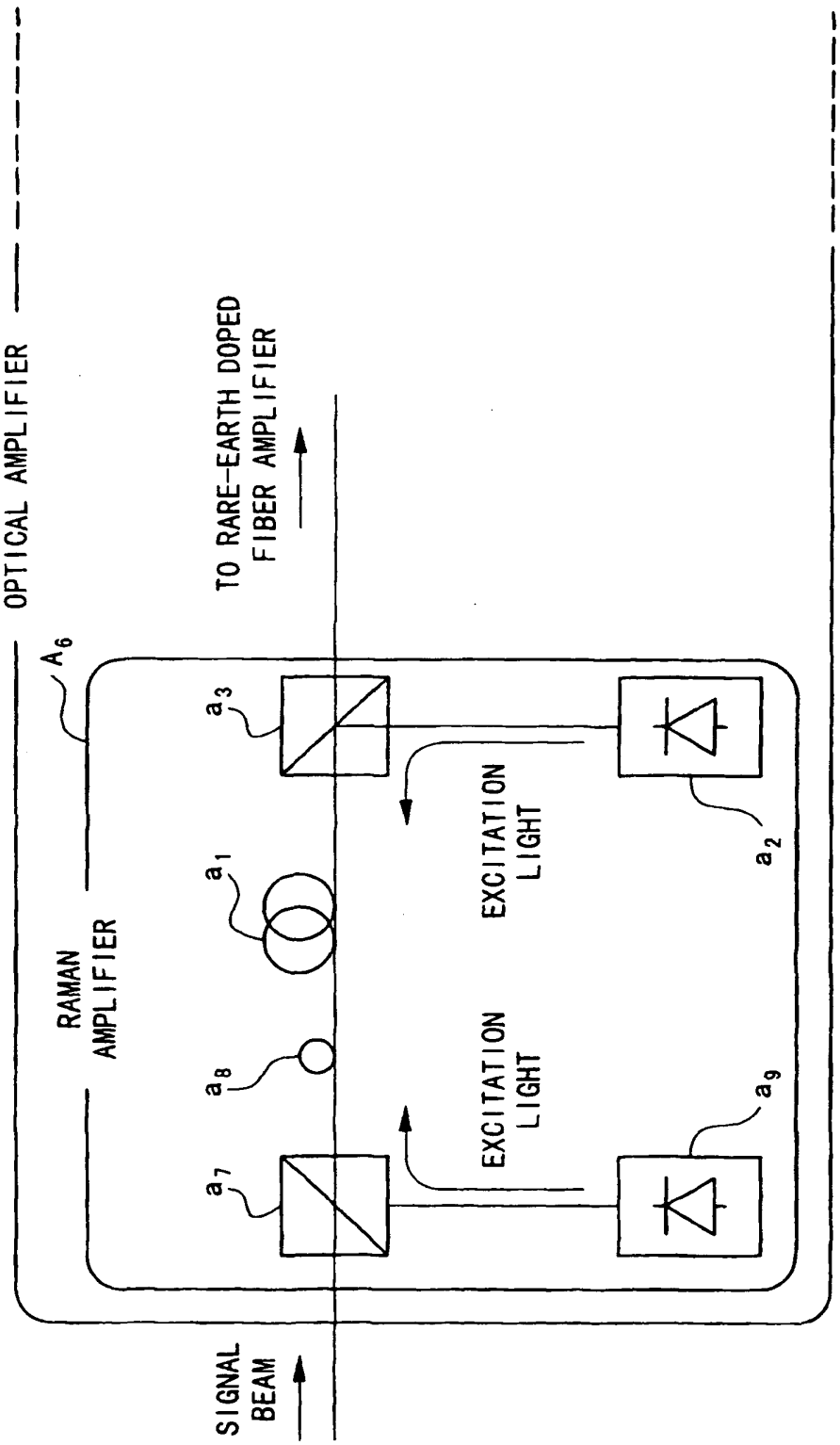

OPTICAL AMPLIFIER
TO RARE-EARTH DOPED FIBER AMPLIFIER
A6
a3
a1
a8
a7
a2
a9
RAMAN AMPLIFIER
EXCITATION LIGHT
EXCITATION LIGHT
SIGNAL BEAM

FIG.34

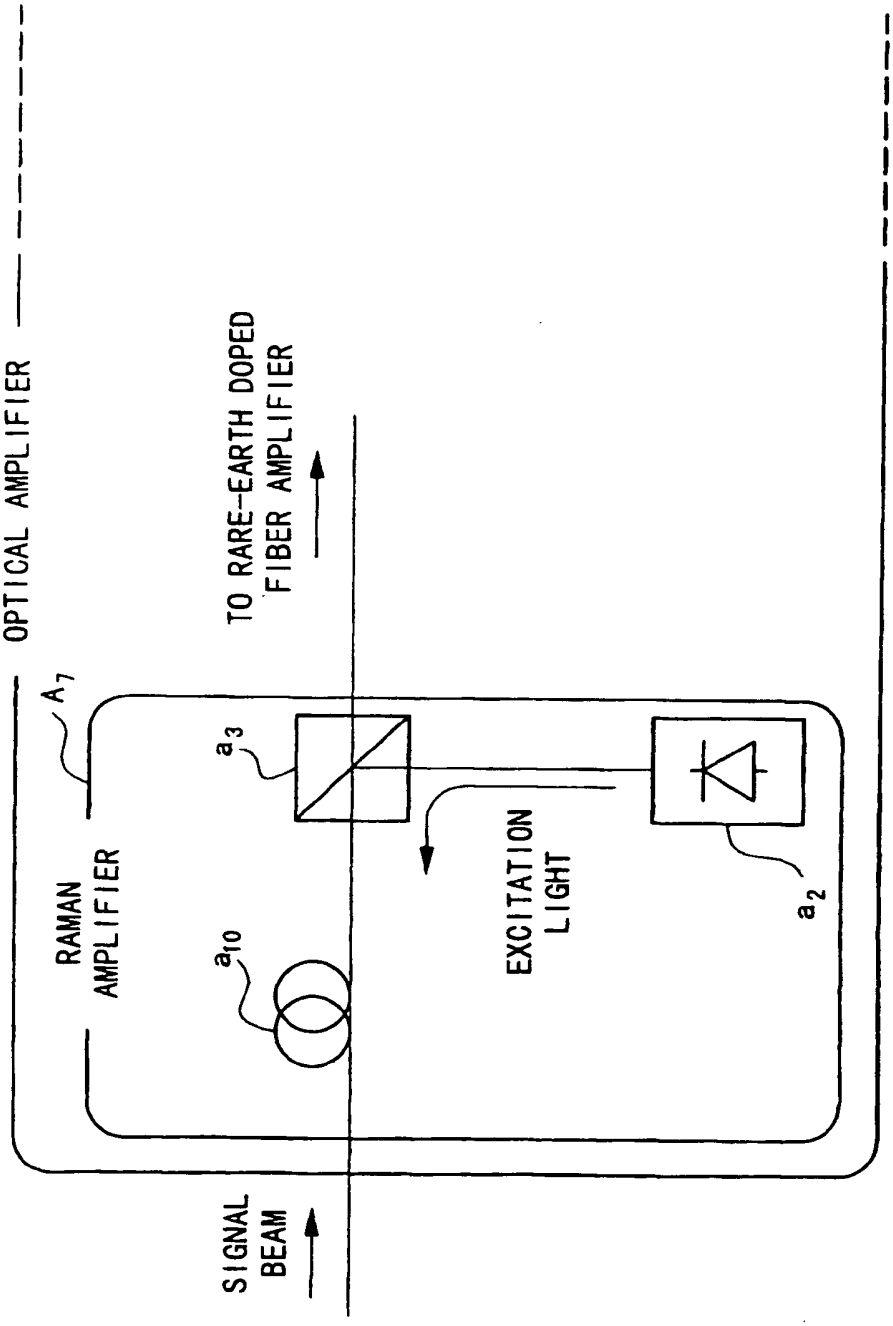
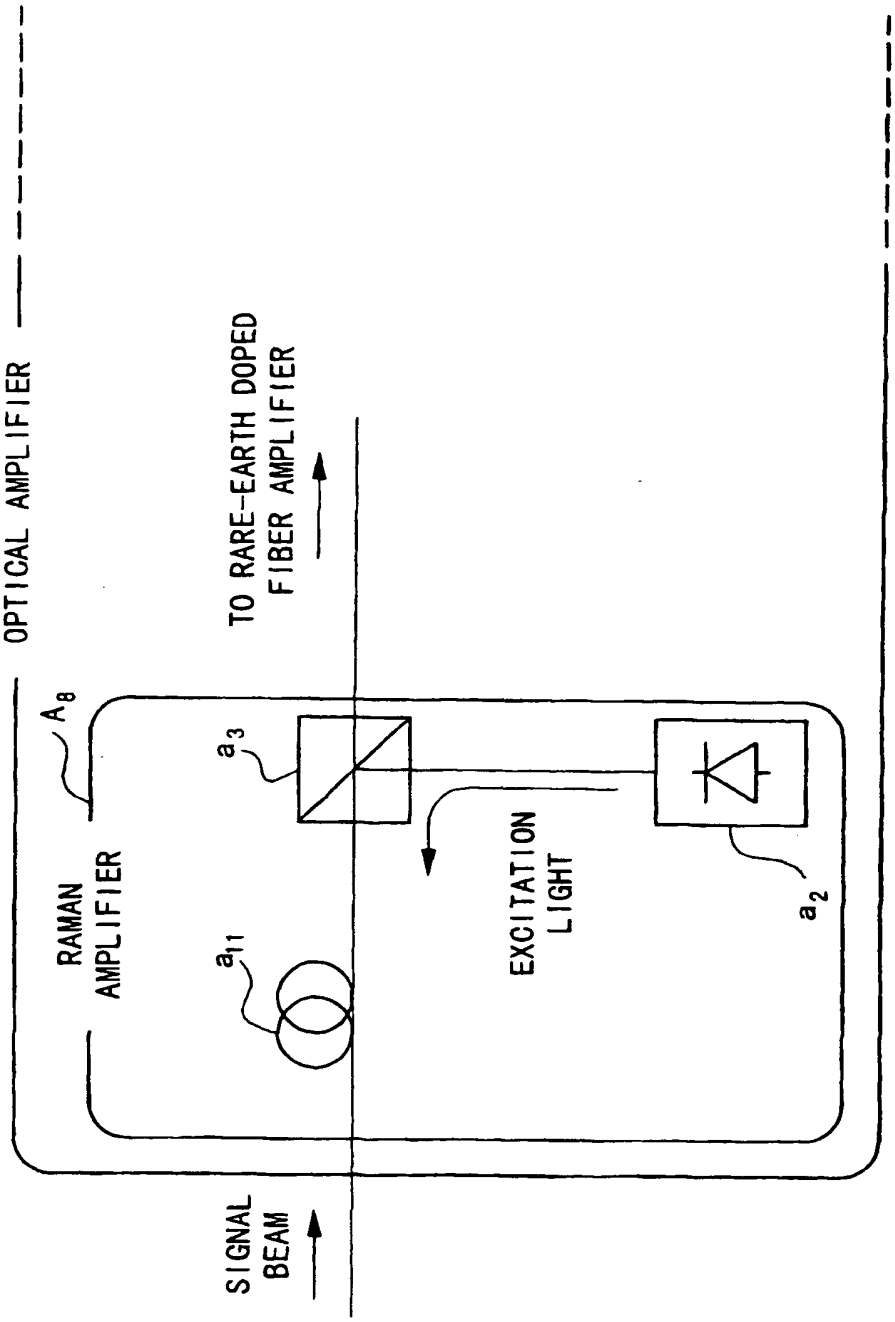

OPTICAL AMPLIFIER
A7
RAMAN AMPLIFIER
a10
a3
TO RARE-EARTH DOPED FIBER AMPLIFIER
EXCITATION LIGHT
a2
SIGNAL BEAM

FIG.35


OPTICAL AMPLIFIER
TO RARE-EARTH DOPED FIBER AMPLIFIER
A_8
a_3
RAMAN AMPLIFIER
a_{11}
EXCITATION LIGHT
a_2
SIGNAL BEAM

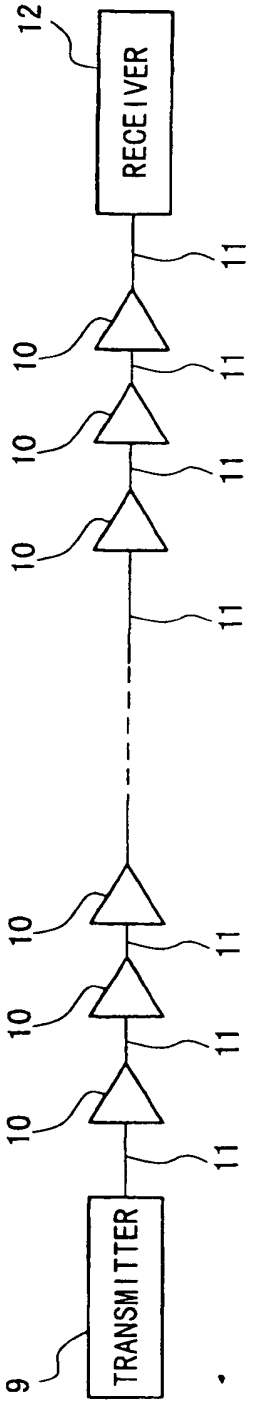


FIG.36A

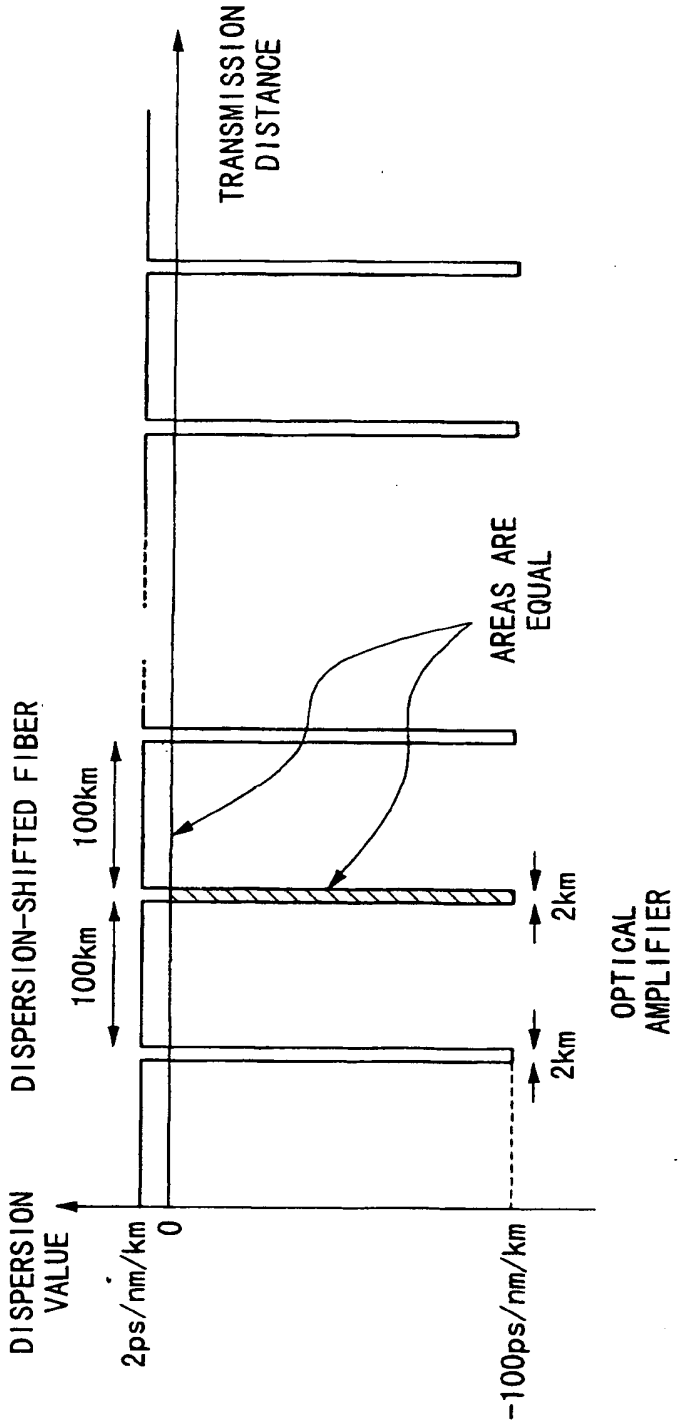


FIG.36B

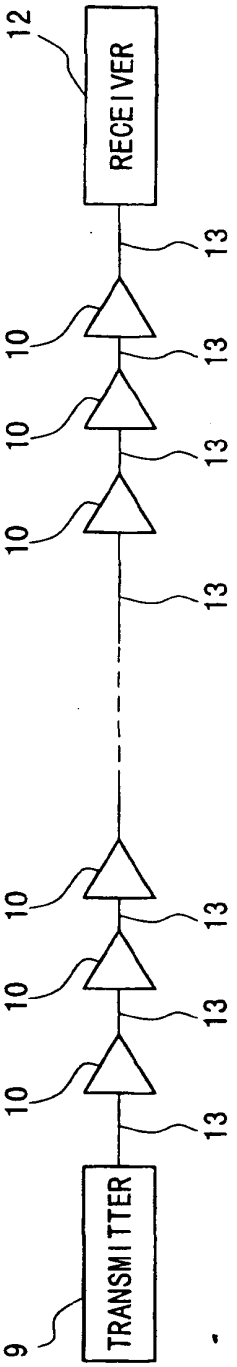


FIG.37A

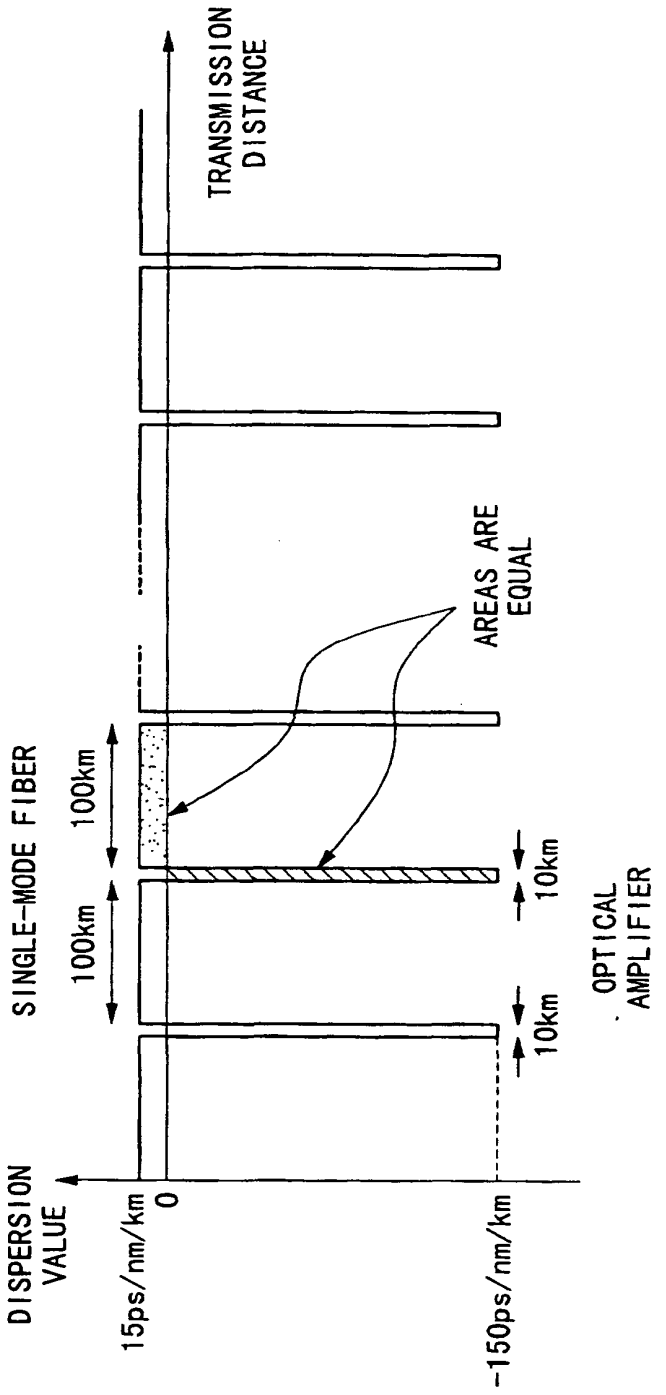


FIG.37B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/00666

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] H01S3/10, H01S3/23, H04B9/00, G02F1/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] H01S3/10, H01S3/23, H04B9/00, G02F1/35

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1940-1996 Toroku Jitsuyo Shinan Koho 1994-1998
Kokai Jitsuyo Shinan Koho 1971-1998 Jitsuyo Shinan Toroku Koho 1996-1998

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JP, 08-213676, A (Oki Electric Industry Co., Ltd.), August 20, 1996 (20. 08. 96), Claims 1 to 3 ; Fig. 1 ; Par. Nos. [0020] to [0028] (Family: none)	1, 3 2, 4-12, 21, 30, 37
Y	JP, 06-077561, A (Toshiba Corp.), March 18, 1994 (18. 03. 94), Fig. 4 ; Par. Nos. [0005] to [0007] (Family: none)	2, 8-12
Y	JP, 01-231030, A (Oki Electric Industry Co., Ltd.), September 14, 1989 (14. 09. 89), Full text (Family: none)	4-7, 13-38
X Y	JP, 06-018945, A (Ando Electric Co., Ltd., Nippon Telegraph & Telephone Corp.), January 28, 1994 (28. 01. 94), Full text (Family: none)	13-15 4-7, 16-38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
May 15, 1998 (15. 05. 98)	May 26, 1998 (26. 05. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/00666

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 06-224505, A (American Telephone and Telegraph Co.), August 12, 1994 (12. 08. 94), Full text & US, 5128800, A	4-7, 13-38
A	JP, 06-169122, A (Matsushita Electric Industrial Co., Ltd.), June 14, 1994 (14. 06. 94), Full text (Family: none)	1-38
A	JP, 07-176817, A (AT & T Corp.), July 14, 1995 (14. 07. 95), Full text & US, 5430572, A & EP, 647000, A1 & AU, 7422494, A1	1-38
E, X	JP, 10-107352, A (Fujikura Ltd.), April 24, 1998 (24. 04. 98), Full text (Family: none)	1-3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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(25) Filing Language: English

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(30) Priority Data:
60/138,969 11 June 1999 (11.06.1999) US

(71) Applicants *(for all designated States except US)*: **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK** [US/US]; Stony Brook, NY 11794-3366 (US). **HSC RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP** [CA/CA]; 555 University Avenue, Toronto, Ontario M5G 1X8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **THOMSEN, Gerald, H.** [US/US]; 201 Bayview Terrace, Port Jefferson, NY 11777-1304 (US). **WRANA, Jeffrey** [CA/CA]; 106B Claremont Street, Toronto, Ontario M6J 2M5 (CA).

(74) Agents: **SEMIONOW, Raina** et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/77168 A2

(54) Title: ANTAGONISTS OF BMP AND TGFβ SIGNALLING PATHWAYS

(57) Abstract: This invention provides unique members of the Hect family of ubiquitin ligases that specifically target BMP and TGFβ/activin pathway-specific Smads. The novel ligases have been named Smurf1 and Smurf2. They directly interact with Smads1 and 5 and Smad7, respectively, and regulate the ubiquitination, turnover and activity of Smads and other proteins of these pathways. Smurf1 interferes with biological responses to BMP, but not activin signalling. In amphibian embryos Smurf1 inhibits endogenous BMP signals, resulting in altered pattern formation and cell fate specification in the mesoderm and ectoderm. The present invention provides a unique regulatory link between the ubiquitination pathway and the control of cell fate determination by the TGFβ superfamily during embryonic development. Thus, Smurf1 is a negative regulator of Smad1 signal transduction, by targeting Smad1, Smurf1 blocks BMP signalling. In mammalian cells, Smurf2 suppresses TGFβ signalling, and in Xenopus, blocks formation of dorsal mesoderm and causes anterior truncation of the embryos. Smurf2 forms a stable complex with Smad7, which induces degradation and downregulation of TGFβ/activin signalling.

ANTAGONISTS OF BMP AND TGFβ SIGNALING PATHWAYS

The research leading to this invention was supported in part by The National Institute of Health Grant No. SR01HD3242902. Thus, the United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

In virtually all animal phyla, critical steps in embryonic development are regulated by cell-to-cell, or inductive, signals mediated by secreted growth factors. In particular, members of the transforming growth factor β (TGFβ) superfamily regulate a myriad of cellular and developmental processes, such as mitosis, cell differentiation, embryonic pattern formation and organogenesis. In vertebrate embryos, a variety of TGFβ signals affect germ layer specification, body patterning, cell growth and differentiation (1-4). In embryos of the amphibian Xenopus, distinct TGFβ members induce different cell fates, *e.g.*, activin, Vg1 and nodal induce mesoderm characteristic of the dorsal part of the embryo, such as notochord and muscle. Vg1 and activin also induce endoderm characteristics. In contrast, Bone Morphogenetic Proteins (BMPs) specify mesoderm, such as blood and mesenchyme, and regulate epidermal and neural cell differentiation in the ectoderm (see (5) for a review).

Cells respond to ligands in the TGFβ family by transducing signals directly from cell surface receptor complexes to nuclear DNA targets via the Smad family of proteins (see (4), (6), (7), and (52) for a review).

Smads are related to *Drosophila* Mad (mothers against *decapentaplegic* [dpp]) and proteins encoded by three related nematode genes Sma 2, Sma 3, and Sma 4. The terms Sma and Mad have been fused as Smad to unify the nomenclature. There are

eight members in the Smad family. Phosphorylated Smads 1, 5 and 8 are functional mediators of BMP family signaling in partnership with Smad 4. Smads 2 and 3 are signal transducers for actions of TGFβ and activins. Smad 6 and Smad 7 function as antagonists to inhibit TGFβ/BMP superfamily signaling. Interestingly, Smad7 is localized in the nucleus and accumulates in the cytoplasm in response to TGFβ signalling (73). Furthermore, expression of both Smad6 and Smad7 is regulated by TGFbs, BMPs, growth factors and cytokines thereby providing for negative feedback regulation of the Smad signalling pathway (53-58). Phosphorylated Smad 1 forms a heteromeric complex with Smad 4 when entering the nucleus and activates transcription of early response genes. The BMP receptors may also signal via the mitogen activated protein kinase. It is likely that BMPs regulate cell cycle progression and thus govern differentiation of mesenchymal stem cells.

Signal transduction in two major pathways, BMP and activin/TGFβ, have been described in detail. Two distinct receptor subunits, the Type I and II transmembrane serine/threonine kinases, form activated complexes upon ligand binding. In these complexes the Type II subunit activates the Type I subunit, which directly phosphorylates and activates particular receptor-regulated R-Smad proteins: BMP receptors target Smad1 and closely-related Smads 5 and 8, while activin and TGFβ receptors target Smad1 and closely-related Smads 2 and 3. Upon activation these R-Smads form a heteromeric complex with Smad4, the "common partner" Smad. This complex translocates to the nucleus, binds to promoters of target genes in cooperation with DNA binding proteins, and activates transcription by recruiting coactivators. A third class of inhibitory Smads (I-Smads), Smad6 and Smad7, function as inhibitors that block Smad-Smad complex formation or Smad-receptor interactions. I-Smads bind to the cytoplasmic domain of receptors or directly to Smad1. Mutations in components at all levels in this pathway are associated with embryonic defects and various cancers, underscoring the importance of this growth factor family in developmental and disease processes. (*See*, (4), (7) for reviews.) In particular, defects of Smad2 and Smad4 are associated with colon and lung cancer and defects in human Smad4 are associated with pancreatic cancer.

Smads do not have intrinsic enzymatic activity. Thus, the nature of the cellular response to Smad signaling is exquisitely sensitive to the level of Smad protein in the cell. Indeed, alternative cell fate determinations in Xenopus embryos can be achieved by relatively small changes in the amount of Smad protein expressed in the cell (8-11).

Therefore, regulating the level of Smad protein in the cell can be used as one means of modulating morphogenetic signaling by the TGFβ superfamily.

Protein modification by covalent attachment of ubiquitin is recognized as a general signal to target proteins for degradation via the proteasome (see (12), and (13) for a review). Targets of selective ubiquitination include transcription factors, cell cycle regulators, signal transduction proteins, and membrane proteins (references in (12)). Selective ubiquitination and degradation of specific target proteins can function as an important mechanism to control cell cycle progression, programmed cell death, differentiation and embryonic development. Dysfunction of the ubiquitination pathway is associated with disease and abnormal development. Ubiquitin ligases are part of a multimeric complex that catalyzes the covalent attachment of ubiquitin, a 12.5 kD polypeptide, to target proteins. Attachment of ubiquitin to its target serves as a molecular "flag" that marks the ubiquitinated protein for proteolytic degradation via an organelle known as the 26S proteosome. There are at least three enzymes involved in conjugating ubiquitin to target proteins, namely, E1, E2 and E3. The E1 enzyme activates a ubiquitin molecule and conjugates it to the E2 enzyme which then either directly attaches ubiquitin onto a target protein, or passes it to the E3 ubiquitin ligase. The E3 recognizes a particular substrate and directs it ubiquitination.

A few examples of developmental regulation by the ubiquitination system have been described in *Dictyostelium* (14-16) and *Drosophila* (17-21). Conjugation of ubiquitin to receptors is used in diverse systems to control endocytosis and signalling, as well as receptor steady state levels by both proteasome- and lysosome-mediated degradation (59-60). Direct ubiquitination of membrane receptors has been characterized in a number of systems, although in some cases ubiquitin-dependent regulation does not appear to involve direct conjugation of ubiquitin to the receptor (61-62). Although many cell surface receptors are regulated by ubiquitin-dependent pathways, few E3 ubiquitin ligases that bind to membrane proteins and target them for ubiquitination have been defined. Nedd4, a C2-WW-HECT domain E3 ubiquitin ligase, can regulate the turnover of the amiloride-sensitive sodium channel by binding directly to a PPXY motif present in the carboxy-terminus of the channel (63-66). Furthermore, the RING finger protein, c-cbl, has recently been shown to function as an E3 ubiquitin ligase that binds to the EGF receptor to mediate ubiquitination and downregulation of the receptor complex (67-68). In these examples, ubiquitination of the membrane proteins appears to involve direct

interactions between the E3 ligase and the target protein. Whether adaptor proteins might also function to recruit E3 ligases to specific receptor complexes is unknown. The mechanism and targets of ubiquitination in the control of patterning have heretofore remained elusive.

References that are cited throughout the specification by number are listed at the end of the Example. All references cited herein are incorporated by reference.

SUMMARY OF THE INVENTION

The present invention advantageously provides a class of regulatory proteins that are involved in BMP and TGFβ-mediated activation. In particular, these proteins regulate Smad proteins and/or promote degradation of TGFβ receptor complexes in the presence of Smad proteins. By manipulating the activity of the proteins of the invention, the skilled artisan can up or down regulate cellular activation, *e.g.*, via BMP or TGFβ.

Thus, in a first aspect, the invention provides an isolated Smurf protein, and particularly a human Smurf protein. In one embodiment, it is a Smurf1 protein. In an alternative embodiment, it is a Smurf2 protein. In specific embodiments, exemplified *infra*, a human Smurf1 has the amino acid sequence depicted in Figure 10 (SEQ ID NO:2), and a human Smurf2 has the amino acid sequence depicted in Figure 12 (SEQ ID NO:4). Smurf proteins of the invention may contain at least about 5 and preferably at least about 10 contiguous amino acids from the sequences depicted in SEQ ID NO:2 and 4.

The invention further provides an antibody that specifically binds to Smurf protein.

The invention further provides a nucleic acid encoding the Smurf proteins of the invention. In specific embodiments, the nucleic acid has a nucleotide sequence as depicted in SEQ ID NO:1 or SEQ ID NO:3.

The invention further provides an oligonucleotide or nucleic acid that specifically hybridizes under highly stringent conditions to a nucleic acid having a sequence encoding Smurf, or the complementary sequence thereof. Such hybridizable nucleic acids include probes (*i.e.*, they may be labeled), primers (*e.g.*, for PCR amplification), anti-sense nucleic acids, ribozymes, and triple-helix forming nucleic acids.

The invention further provides a vector comprising the nucleic acid encoding Smurf, *e.g.*, under control of an expression control sequence. Also provided are host cells, harboring such a vector, and methods for producing Smurf by culturing such host cells under conditions that permit expression of Smurf protein from the vector.

Also contemplated is a transgenic non-human animal that expresses a human Smurf protein and non-human animals in which endogenous Smurf protein is deleted.

The invention further provides a method for inhibiting a bone morphogenic protein or transforming growth factor-beta activation pathway in a cell. This method comprises permitting the cell to grow under conditions that permit expression of Smurf from a vector of introduced into the cell. Alternatively, the invention provides a method for promoting a bone morphogenic protein or transforming growth factor-beta activation pathway in a cell, which method comprises suppressing expression of endogenous Smurf in the cell.

In addition, the present discoveries permit screening for a modulator of Smurf activity. Screens of the invention comprise detecting modulation of Smurf activity in the host cell in the presence of a test compound relative to Smurf activity of the host cell in the absence of the test compound. As shown in the examples, one such activity is ubiquitination of Smad proteins. Another activity is the enhancement of TGFβ receptor degradation.

DESCRIPTION OF THE DRAWINGS

Figure 1. *Smurf1 encodes an E3 ubiquitin ligase.* Protein sequence of Xenopus and human Smurf1 compared to yeast (*S. pombe*) pub1, given as SMURF1, hSMURF1 and PUB1, respectively in the figure. Identical amino acids are shaded dark gray and conservative substitutions are shaded light gray. Based on primary structure Smurf1 and pub1 are members of the Hect family of E3 ubiquitin ligases and display several conserved features of the family: A lipid/Ca2+ binding domain is located at the N-terminus (residues 22-37), two WW protein interaction domains at 236 - 271 and 282 -311 (indicated by thick lines) , and a catalytic Hect domain beginning at residue 347 and extending to the C-terminus. Alignment was by Clustal W analysis (MacVector).

Figures 2A and 2B. *Northern blots of mSmurf1 expression in embryonic and adult mouse tissues* . Equal amounts of PolyA+ mRNA from indicated stages and tissues were analyzed. **(2A)** Embryonic tissue - In this blot, embryonic days post coitum are indicated. **(2B)** Adult tissues shown are T, testes; K, kidney; M, skeletal muscle; L, lung; Sp, spleen; Br, brain; H, heart.

Figures 3A and 3B. *Developmental expression of Xenopus Smurf1.*

(A) RT-PCR on staged embryonic cDNA revealed that Xenopus Smurf1 is a maternal mRNA, present at highest levels in egg, blastula and early gastrula stages. Zygotic Smurf1 mRNA levels decline at gastrulation but maintain steady expression into

swimming tadpole stages. Numbers above each lane correspond to Nieuwkoop and Faber stages: 7 and 9, blastula; 11 and 13, gastrula; 15 and 20, neurula; 25 and 35, tadpole. Ornithine decarboxylase (ODC) mRNA, ubiquitously expressed in cells, was assayed to normalize for RNA recovery. RT-PCR on mock cDNA (no reverse transcriptase).

(B) Whole-mount in situ hybridization of Smurf1 in developing Xenopus embryos. In egg and blastula stages Smurf1 transcripts are localized to the animal pole half (bracket in egg). Expression is diffuse throughout the ectoderm and involuting marginal zone of the gastrula; views from , animal pole (an) and vegetal pole (Veg). There is some enrichment of transcripts in the neural folds at neurula stage 17. At tadpole stages 25 and 35 Smurf1 expression includes the brain (b), eye (e), otic vesicle (o), somites (s), pharyngeal pouches (p) and developing kidney (k).

Figures 4A, 4B, 4C, and 4D. *Smurf1 leads to a selective decrease in steady-state level of Smad1 and Smad5 in mammalian cell lines.* Cells were transiently transfected with the indicated expression vectors (DNA quantities in μg) and two days later an aliquot representing approximately 0.4% of total cell lysates were subjected to SDS-PAGE and immunoblotting. To determine the steady-state level of the Smads, the blots were probed with the appropriate Smad antibody as shown. Flag-hSmurf1 expression level was confirmed by probing blots of total cell lysates with the anti-Flag monoclonal antibody.

(A) COS-1 or 293T-cells were transfected with a constant amount of pCMV5-Smad1 and increasing concentrations of pCMV5-Flag-hSmurf1 as indicated. To determine Smad1 steady-state level and the expression of hSmurf1, Western blots of total cell lysates from both cell lines were probed with α-Smad1 and α-Flag antibodies (α-Smad1 and α-Flag blot).

(B) 293T cells were transiently transfected with pCMV5-Smad1, wild type or activated (Q203D) pCMV5-ALK6-HA, and increasing amounts of pCMV5-Flag-hSmurf1 as indicated. Smad1 steady-state levels were examined by immunoblotting total cell lysates in Western blots with α-Smad1 antibody (α-Smad1). hSmurf1 and wild type or activated ALK6 expression levels were determined by immunoblotting the total cell lysates with α-Flag (α-Flag) or α-HA (α-HA) antibody, respectively.

(C) 293T cells were transfected with a constant amount of pCMV5-Smad1 or pCMV5-Smad2 along with the indicated concentrations of pCMV5-Flag-hSmurf1. Smad1 (α-Smad1 blot), Smad2 (α-Smad2 blot) and Flag-hSmurf1 (α-Flag blot) steady-state protein levels were determined in cell lysates, as above.

(D) 293T cells were transfected with Smad1, Smad3, Smad4 or Smad5 in pCMV5 in the absence or presence of pCMV5-Flag-hSmurf1. The steady-state level of the Smads in total cell lysates was determined by incubating the Western blots with α-Smad1 antibody for Smad1 and Smad5, α-Smad3 antibody for Smad3, and α-Smad4 antibody for Smad4 detection (α-Smads blot). Equivalent hSmurf1 expression was confirmed as described above (α-Flag blot).

Figures 5A, 5B, and 5C. *hSmurf1 regulates Smad1 turnover and ubiquitination: hSmurf1 enhances Smad1 turnover.*

(A) COS-1 cells were transiently transfected with pCMV5-Smad1 alone or with Flag-tagged hSmurf1 (F/hSmurf1) using lipofectAMINE. Two days later, transfectants were subjected to pulse-chase analysis using [^{35}S]methionine. At the indicated time during the chase, cells were lysed and subjected to an α-Smad1 immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography (left panel). Radiolabelled Smad1 was also quantitated by phosphoimaging, and the results are plotted as the amount of [^{35}S] methionine-labelled Smad1 present at each time point relative to the level at time 0 (right panel).

(B) Ubiquitination of Smad1 in 293T cells. Cells were transiently transfected with indicated combinations of HA-tagged ubiquitin (HA-Ub), pCMV5-Smad1 and either wild type (WT) or the ubiquitin ligase mutant (CA) of Flag-tagged hSmurf1. Two days post-transfection, lysates were subjected to an α-Smad1 immunoprecipitation (α-Smad1 IP) followed by SDS-PAGE and immunoblotting with an α-HA monoclonal antibody (α-HA blot). Protein bands displaying immunoreactivity to α-HA are marked by the square bracket. Expression of Smad1 or Flag-hSmurf1 was confirmed by subjecting total cell lysates to immunoblotting with an α-Smad1 polyclonal antibody (α-Smad1 blot) or an α-Flag monoclonal antibody (α-Flag blot), respectively.

(C) Loss in Smad1 steady-state level by hSmurf1 requires an intact ubiquitin ligase activity of the Hect domain. 293T cells were transfected with Smad1 and increasing amounts of the wild type (WT) or the ubiquitin ligase mutant (C710A). Total cell lysates were analyzed for Smad1, hSmurf1 or hSmurf1(C710A) protein by immunoblotting total cell lysates with the appropriate antibody, as described in Fig 3.

Figures 6A, 6B, and 6C. *Interaction of Smurf1 and Smads.*

(A) Smurf1 interacts with Smad1. Yeast two-hybrid assays were done on yeast co-transformed with combinations of Xenopus Smurf1 (Xsmurf) together with Smad1, Smad2, lamin or vector alone. Only the combination of Smad1 and Smurf1 exhibited significant β-galactosidase activity (left panel, photograph of stained yeast colonies assayed in duplicate). Co-immunoprecipitation on *in vitro* translated proteins was

done by incubating 35S-labelled Smurf1 together with *in vitro* translated, 35S-Met trace-labelled Flag-tagged Smad1 (35S-F/Smad1), Smad2 (35S-F/Smad2) or Smad4 (35S-F/Smad4) immobilized on anti-Flag affinity gel matrices. After washing, bound proteins were eluted and analyzed by SDS-PAGE (right panel).

(B) hSmurf1 interacts selectively with both Smad1 and Smad5. 293T cells were transiently transfected with the pCMV5 expression vectors containing Smad1 (S1), Smad5 (S5) or Smad2 either alone, with wild type (WT) or the ubiquitin ligase mutant (CA) Flag-tagged hSmurf1 (F/hSmurf1). To examine Smad1 or Smad5 interaction with hSmurf1, blots of α-Flag immunoprecipitates were probed with α-Smad1 polyclonal antibody (α-Smad1 blot). To examine Smad2 interaction with hSmurf1, blots of α-Flag immunoprecipitates were probed with α-Smad2 polyclonal antibody (α-Smad2 blot). The level of hSmurf1 in the immunoprecipitates and Smad1, Smad5 and Smad2 in the total cell lysates, are shown in the lower two panels.

(C) Specificity of hSmurf1 actions on Smad1. Nedd4, a ubiquitin ligase related to hSmurf1, does not interact with and does not reduce the steady-state level of Smad1. 293T cells were transiently co-transfected with Smad1 and either Flag-tagged hSmurf1 (WT or CA) or Nedd4, (WT) or ligase mutant (CS) as indicated. Smad1 coimmunprecipitation with hSmurf1 (left panels) was determined as described above. To determine Smad1 interaction with Nedd4, cell lysates were subjected to immunoprecipitation using a-Hect-Nedd4 polyclonal antibody (α-Nedd4 IP) followed by immunoblotting with α-Smad1 polyclonal antibody (α-Smad1 blot), right panels. To determine the level of expression of Nedd4 in the samples, the respective blots were reprobed with a-WW2 Nedd4 polyclonal antibody (α-Nedd4 blot). Smad1 levels in total cell lysates of these assays are shown by western blot in the bottom panel.

Figures 7A and 7B. *Smurf1 dorsalizes prospective ventral mesoderm and neuralizes ectoderm.*

(A) Four cell Xenopus blastulae were injected with Xenopus Smurf mRNA in the marginal zone of two ventral blastomeres (50 pg Smurf1 mRNA per cell). Tadpoles that developed from injected embryos formed ectopic, dorsal axial structures (lower left panel, arrows). Co-injection of 100 pg Smad1 together with 50 pg of Smurf1 mRNA rescued the ectopic axial structures in all cases (lower tadpole). At early gastrulation ventral marginal zone (VMZ) pieces were explanted from another set of wild-type embryos, or embryos injected in the VMZ at the four cell stage with Smurf1mRNA alone, or Smurf1 co-injected with Smad1. The VMZ explants were excised at early gastrulation, as depicted, then cultured until control embryos reached mid-tadpole, stage 28, when total VMZ RNA was prepared then assayed by RT-PCR for the expression of

erythrocyte-specific α-globin, muscle-specific actin, and general cellular mRNA, eF1-a, as a control for RNA recovery (lower right panel). Control PCR reactions were done on total embryonic RNA with or without reverse-transcription.

(B) Fertilized egg animal poles were injected with Smurf1 and/or Smad1 mRNA, or were not injected. Animal caps were explanted at blastula stage 8, cultured to mid-gastrula stage 11, then total RNA was prepared and assayed by RT-PCR to detect expression of the cement gland marker, XAG, and a general neural marker, NCAM. Controls were as above.

Figures 8A, 8B, and 8C. *Smurf1 alters embryonic cell competence to respond to Smad1 and Smad2.*

(A) Smurf1 blocks ventral mesoderm induction by Smad1. A constant amount (1 ng) of Smad1 mRNA was injected alone, or together with an increasing amount of Smurf1, into fertilized egg animal poles and ventral mesoderm induction in animal cap explants was assayed by RT-PCR with primers for the Xhox3 and Xcad3 homeodomain genes. In lanes from left to right, respectively, animal caps were not injected or injected with Smurf1 mRNA at doses of 100, 0, 25, 50, 100, and 200 pg.

(B) Smurf1 enhances dorsal mesoderm induction by Smad2. A constant amount (50 pg) of Smad2 mRNA was injected alone, or together with an increasing amount of Smurf1, and dorsal mesoderm induction was monitored by expression of myoD, which marks muscle, and goosecoid, a homeodomain gene expressed in the most dorsal type of mesoderm, the Spemann Organizer. Note that goosecoid expression was triggered from undetectable levels, as the dose of Smurf1 was increased. Animal caps were injected as in panel a.

(C) Dose-response of animal caps to Smad2. Animal caps were injected with synthetic mRNAs for Smad2. At 50 pg Smad2 MyoD was induced and reached maximal levels at 100 pg or more injected Smad2. Goosecoid was induced at a minimum Smad2 dose of 250 pg. The level of goosecoid induced by 250 pg Smad2 alone was equivalent to the level of goosecoid induced by a combination of 50 pg of Smad2 and 100 pg of Smurf1 (panel b).

In all panels the far right two lanes correspond to PCR on wildtype embryonic RNA, with (RT+) and without (RT-) reverse transcription, respectively. eF1-a controls in all panels were as in **Fig. 7**.

Figure 9. *cDNA sequence of human Smurf1* **[SEQ ID NO: 1]**.

Figure 10. *Protein sequence of human Smurf1* **[SEQ ID NO:2]**.

Figure 11. *cDNA sequence of human Smurf2* **[SEQ ID NO: 3]**.

Figure 12. *Protein sequence of human Smurf2* **[SEQ ID NO: 4]**.

Smurf2 is member of the HECT E3 ubiquitin-ligase family. The C2 (overline), WW (shaded) and HECT (double overline) domains and the Cys716Ala mutation (boxed) are shown.

Figure 13. *Comparison of Smurf1 and Smurf2 proteins.*

Schematic representation of Smurf1 and Smurf2. The degree of amino acid identity (%) between the C2, WW and HECT domains is shown.

Figures 14A and 14B. *Smurf2 is expressed in mouse tissues.*

(A) Smurf2 is expressed throughout mouse embryogenesis. A 1 kb XhoI/NotI fragment encompassing the 3'UTR of mouse Smurf2 was used to probe a mouse embryonic Northern Blot (Clontech).

(B) Expression of Smurf2 in adult mouse tissues. A multiple tissue northern blot (Clontech) was probed with a fragment of mouse Smurf2 as in A.

Figures 15A, 15B, 15C, 15D, 15E and 15F. *Smurf2 interacts with Smad7.*

(A and B) Expression of Smurf2 does not decrease steady-state levels of the Smads. 293T cells were transfected with Flag-tagged Smad1, Smad2, Smad4 or HA-tagged Smad7 either alone or together with the Myc-tagged Smurf2. Aliquots of total cell lysates were immunoblotted to detect expression of Smurf2 and the Smads (**Fig. 15A**) or were subjected to immunoprecipitation with anti-Myc antibody followed by anti-Flag or anti-HA immunoblotting to detect any coprecipitating Smads (**Fig. 15B**). The migration of the anti-Myc heavy chain (IgH) is marked.

(C) Expression of Smurf2 does not alter Smad7 turnover. COS-1 cells, transfected with either Smad7-HA alone or together with Flag-Smurf2, were pulse-labelled with [^{35}S]-methionine and then chased for the indicated times in media containing unlabelled methionine. [^{35}S]-labelled Smad7-HA in anti-HA immunoprecipitates was quantified by phosphorimaging and the levels in control cells (squares) and Smurf2 expressing cells (circles) was plotted relative to the amount present at time 0. Data represents the average of two experiments +/- SD.

(D) *In vitro* interaction of bacterially-expressed Smurf2 and Smad7. Bacterially-produced His-Smad7-HA protein was incubated with Ni^{2+}-NTA, GST and GST-Smurf2. Bound material was visualized by SDS-PAGE and immunoblotting with anti-HA antibody. Levels of GST proteins were determined by Coomassie staining (bottom panel).

(E) The PY motif in Smad7 is an important determinant for mediating interaction with Smurf2. 293T cells were transfected with Flag-Smurf2 either alone or together with wild type (WT) or mutant Y211A (YA) or ΔPYversions of Smad7-HA. Cell lysates were subjected to anti-Flag immunoprecipitation and coprecipitating Smad7

proteins were detected by immunoblotting with anti-Smad7 antibody. Smad7 expression was confirmed by immunoblotting aliquots of total cell lysates (bottom panel).

(F) The WW domains of Smurf2 are necessary for binding to Smad7. 293T cells were transfected with Smad7-HA and either wild type (WT) or mutant (ΔWW1, ΔWW2 or ΔWW3) versions of Flag-Smurf2. Cell lysates were subjected to anti-Flag immunoprecipitation, and coprecipitating Smad7 was detected by immunoblotting with anti-HA antibody. Smad7 expression was confirmed by immunoblotting aliquots of total cell lysates (bottom panel).

Figure 16. *Smad7 recruits Smurf2 to the TGFβ receptor complex.* COS-1 cells were transfected with various combinations of TβRII, TβRI-HA, Smad7-HA and wild type (WT) or mutant (C716A) Flag-Smurf2 as indicated. Cells were affinity-labelled with [^{125}I]TGFβand lysates immunoprecipitated with anti-Flag or anti-Smad7 antibodies. Coprecipitating receptor complexes were visualized by SDS-PAGE and autoradiography. The amount of coprecipitating TβRI was quantified by phosphorimaging (right panels). Receptor expression was confirmed by visualizing aliquots of total cell lysates by autoradiography. Smurf2 and Smad7 levels were confirmed by immunoblotting aliquots of total cell lysates with anti-Flag and anti-HA antibodies, respectively.

Figures 17A, 17B, 17C, 17D and 17E. *Smurf2 induces degradation of TGFβreceptors and Smad7.*

(A) *Smurf2 expression in the absence of Smad7 does not decrease receptor steady-state levels.* 293T cells were transfected with various combinations of TβRII-HA, TβRI-HA and varying amounts of Flag-Smurf2 (plasmid DNA in micrograms) is indicated. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates using the appropriate antibodies as shown.

(B) *Smurf2 in the presence of Smad7 causes a decrease in steady-state receptor levels.* 293T cells were transfected with Smad7-HA, either TβRII-HA and TβRI-HA (left panels) or with a constitutively active type I receptor, TβRI-HA (T204D) (right panels) together with increasing amounts of wild type (WT) or mutant Flag-Smurf2 (C716A). Steady-state levels of the receptors, Smad7 and Smurf2 were determined by anti-HA or anti-Flag immunoblotting as indicated.

(C) *Smurf2 increases the turnover rate of the receptor complex.* COS-1 cells transfected with TGFβreceptors (TβRII-HA and TβRI-HA) alone or together with Smad7-HA, Flag-Smurf2 or both were pulse-labelled with [^{35}S]-methionine and then chased for the indicated times in media containing unlabelled methionine. Cell lysates were subjected to anti-HA immunoprecipitation and the amount of labelled receptors and

Smad7 was quantified by phosphorimaging and is plotted relative to the amount present at time 0.

(D) *Proteasome and lysosome inhibitors block Smurf2-induced degradation of the receptor complex.* COS-1 cells transfected with TGFβ receptors (TβRII-HA and TβRI-HA), Smad7-HA and Flag-Smurf2 were pulse-labelled with [^{35}S]-methionine and then chased either in absence of inhibitors or in the presence of 30mM lactacystin, or 0.4 mM chloroquine for the indicated times. Cell lysates were subjected to anti-HA immunoprecipitation and receptor and Smad7 levels were visualized by SDS-PAGE and autoradiography.

(E) *Smurf2 induces the ubiquitination of Smad7 in the presence of the receptors.* 293T cells were transfected with HA-tagged ubiquitin together with various combinations of Smad7, TβRII, TβRI-Flag, and wild type (WT) or mutant (C716A) Myc-Smurf2 as indicated. Cell lysates were subjected to a double immunoprecipitation with anti-Smad7 antibodies followed by immunoblotting with anti-HA antibodies. Protein expression in aliquots of total cell lysates was confirmed by immunblotting.

Figures 18A, 18B, 18C and 18D. *Association of Smurf2 with Smad7 enhances the Smad7 inhibitory activity.*

(A) *Smad7(Y211A) binds to TGFβ receptors but has a reduced ability to recruit Smurf2 to the receptor complex.* COS-1 cells were transfected with TGFβ receptors (TβRII and TβRI-HA) and either wild type (WT) or mutant (Y211A) Smad7/HA in the absence or presence of Flag-Smurf2(C716A). Cells were affinity-labelled with [^{125}I]TGFβ and lysates immunoprecipitated with anti-Smad7 or anti-Flag antibodies. Coprecipitating receptor complexes were visualized by SDS-PAGE and autoradiography. Total receptor expression was determined by autoradiography and Smad7 and Smurf2 protein levels were confirmed by anti-HA or anti-Flag immunoblotting of aliquots of total cell lysates.

(B and C) *Smad7(Y211A) is not as effective as wild type Smad7 in inhibiting TGFb-dependent activation of transcription.* HepG2 cells were transfected with the 3TP-Lux reporter alone or together with varying concentrations of wild type (WT) or mutant (Y211A) Smad7-HA. In **(B)**, 0.3 ng/ml of each Smad7 plasmid was used. Cells were incubated in the presence or absence of TGFβ1, and luciferase activity was normalized to b-galactosidase activity and is plotted as the mean +/- SD of triplicates from representative experiments.

(D) *A model for Smad7 and Smurf2-mediated degradation of the TGFβ receptor complex.* Smad7 binds directly to Smurf2 and associates with the TGFβ receptor

complex. Thus, Smad7 functions as an adaptor protein that mediates degradation of the TGFβ receptor complex.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a family of genes encoding E3 ubiquitin ligases, called Smurf proteins, and includes full length, or naturally occurring forms, and any functionally active or antigenic fragments thereof, from any animal, particularly mammal or amphibian, and more particularly from a human source. In specific embodiments, the E3 ubiquitin ligases called Smurf1 and Smurf2 are characterized.

This invention is based, in part, on the surprising discovery that two novel members of the Hect family of ubiquitin ligases interact with Smads. These ligases have been named Smurf1 and Smurf2. One of these, Smurf1, specifically targets BMP pathway-specific Smads, thereby acting as an antagonist or negative regulator of BMP signaling. Smurf1 directly interacts with Smads1 and 5 and regulates their ubiquitination, turnover, and activity. In amphibian embryos, Smurf1 inhibits endogenous BMP signals, resulting in altered pattern formation and cell fate in the mesoderm and ectoderm. Thus, the invention provides a unique regulatory protein link between the ubiquitination pathway and the control of cell fate, *e.g.*, during embryonic development. The invention further provides a nucleic acid encoding Smurf1 **[SEQ ID NO: 1]**, in addition to the Smurf1 protein, **[SEQ ID NO: 2]**, and mutant variants thereof.

The other novel member of the Hect family of ubiquitin ligases is Smurf2. Smurf2, a C2-WW-HECT domain E3 ubiquitin ligase is related to Smurf1. Smurf2 does not interact with Smad1, 2 or 4, nor does Smurf2 alter steady state levels of Smad 1, 2 or 4. Smurf2, however, does interact with Smad 7, binding directly to a PPXY motif in Smad7. Smurf2 is involved with TGFβ receptor degradation acting in partnership with Smad7 as an antagonist or negative regulator of TGFβ signaling. Activation of TGFβ signalling results in Smad7-dependent recruitment of Smurf2 to the TGFβ receptor complex. In the absence of activated TGFβ receptor complex, Smurf2 does not alter the steady state level and turnover of Smad7. Recruitment of Smurf2 to the TGFβ receptor by Smad7 promotes the degradation of the Smad7- TGFβ receptor complex by both proteasomal and lysosomal pathways. The studies described herein demonstrate that Smad7 functions as an adaptor protein that recruits Smurf2 to the TGFβ receptor complex to promote its degradation and thereby down-regulate activated TGFβ receptor complexes. Regulation of Smad 7 localization to the nucleus and interaction with Smurf2 may be used to control the inhibitory activity of the Smad 7-Smurf2 complex.

The invention further provides a nucleic acid encoding Smurf2 **[SEQ ID NO: 3]**, in addition to the Smurf 2 protein **[SEQ ID NO: 4]** and variants thereof.

E3 ubiquitin ligases display very selective substrate specificity, as is evident in the findings discussed herein. For example, Smurf1 binds BMP pathway-specific Smads. Moreover, Smurf1 is a unique signaling protein of the BMP pathway because it binds only to Smad1 and Smad5 and has little affinity for Smad 2 (specific for TGFβ and activin receptor pathways), and no affinity for Smad4 (common Smad signaling partner). As a result of this substrate specificity, Smurf1 can effectively interfere or regulate biological responses to BMPs without consequence to activin pathways, *i.e.*, effects on other TGFβ signaling pathways are limited, or non-existent.

BMPs/TGFβ signalling pathway functions in tissue differentiation, morphogenesis, and cell growth control (*e.g.*, (52)). As an antagonist to the signal transduction pathway mediated by the TGFβ family, Smurf1 will inhibit the BMP pathway *in vivo* or *in vitro*. As an integral component of regulatory system for degradation of TGFβ receptors, Smurf2 will inhibit the TGFβ signalling pathway. As a result, the Smurfs will block chondrogenesis, osteogenesis, blood differentiation, cartilage formation, neural tube patterning, retinal development, heart induction and morphogenesis, hair growth, tooth formation, gamete formation and a wide variety of tissue and organ formation processes, and hinder the regeneration, growth, maintenance, etc., of bone and other tissues that are dependent on the BMP pathway.

In one embodiment, mutant forms of a Smurf protein or small molecule antagonists of Smurf, described *infra*, can be used to prevent ubiquitination of proteins, *e.g.*, Smads or TGFβ receptor, and therefore to preserve the signal transduction pathway mediated by BMPs. In addition, fragments of Smads can be generated that bind to either the Hect domain of a Smurf protein, which domain has the catalytic activity needed for ubiquitination of Smads, or to the WW domains that interact with the PPXY domains of Smads, thus precluding Smurf1 from binding and ubiquitinating Smads. These fragments can also be used in screening assays for small molecule inhibitors of Smurf-Smad interaction, *e.g.*, in an inhibition binding assay. Variants of a Smurf protein and fragments of Smads can be used to improve defective BMP and/or TGFβ/activin signaling as a result of overexpressed or increased Smurf activity, which may contribute to a disease state.

BMPs control bone differentiation and growth, and are already in clinical tests and applications related to bone growth and connective tissue repair. Smurf proteins represent a novel target for the discovery of drugs that can influence its function, thereby affecting cellular responses to BMPs, and thus having clinical applications. Therefore, a Smurf protein can be used for screening for various drugs and/or antibodies that can either

enhance the BMP pathway, or inhibit it by antagonizing or mimicking the activity of the protein, respectively. For example, since Smurf1 is highly specific for binding Smads1 and 5, it can be used to screen for drugs that block or activate the BMP pathway, and selectively affect cellular responses to BMPs without consequence to other members of the TGFβ superfamily.

Smurf proteins operate within cells, at the level of Smad signal transduction, and therefore provide an alternative means to affect BMP and TGFβ/activin signals. However, because Smurf proteins are intracellular proteins, any manipulations aimed at directly altering Smurf activity must operate intracellularly. Such manipulations include antisense and ribozyme technology, and intracellular antibody technology.

Smurf may be delivered to cells in gene therapy regimens to block excessive signaling by particular growth factor pathways controlled by Smads, *e.g.*, Smads that are targets for Smurf1 or Smurf2. The examples herein show that simply increasing the expression levels of Smurf1 in cells antagonizes the Smad signaling pathway. Thus overexpression of Smurfs by gene therapy may be used to correct clinical conditions that result from excessive Smad signaling. These may include, for example, hyperplasia of bone, tendon or cartilage tissues, or formation of other tissues, that are regulated by signals from receptors (such as BMP receptors) that utilize Smad1 or Smad5 for signal transduction.

Smurf nucleic acids or partial sequences thereof (such as PCR probes) would be useful as molecular probes for identification of defective Smurf genes in the human genome, particularly where a mutation of a Smurf gene is found in association with a particular disease. Smurf proteins may be used as reagents for *in vitro* assays to identify proteins in cells that are targets for ubiquitination. Purified Smurfs may be reconstituted with purified ubiquitination enzymes (*i.e.* E1 and E2 components) and utilized in functional (ubiquitination) assays that are aimed at identifying novel target proteins introduced into the assays (as purified proteins or translated cDNAs of unknown identity).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed.

(1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

If appearing herein, the following terms shall have the definitions set out below.

The term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

As used herein, the term "isolated" means that the referenced material is free of components found in the natural environment in which the material is normally found. In particular, isolated biological material is free of cellular components. In the case of nucleic acid molecules, an isolated nucleic acid includes a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. In another embodiment, an isolated nucleic acid is preferably excised from the chromosome in which it may be found, and more preferably is no longer joined to non-regulatory, non-coding regions, or to other genes, located upstream or downstream of the gene contained by the isolated nucleic acid molecule when found in the chromosome. In yet another embodiment, the isolated nucleic acid lacks one or more introns. Isolated nucleic acid molecules can be inserted into plasmids, cosmids, artificial chromosomes, and the like. Thus, in a specific embodiment, a recombinant nucleic acid is an isolated nucleic acid. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein. An isolated organelle, cell, or tissue is removed from the anatomical site in which it is found in an organism. An isolated material may be, but need not be, purified.

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate unrelated materials, *i.e.*, contaminants. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is associated in a cell; a purified nucleic acid molecule is preferably substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more preferably, at least 90% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, and other methods known in the art.

A "gene" is used herein to refer to a portion of a DNA molecule that includes a polypeptide coding sequence operatively associated with expression control

sequences. In one embodiment, a gene can be a genomic or partial genomic sequence, in that it contains one or more introns. In another embodiment, the term gene refers to a cDNA molecule (*i.e.*, the coding sequence lacking introns).

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

"Expression control sequences", *e.g.*, transcriptional and translational control sequences, are regulatory sequences that flank a coding sequence, such as promoters, enhancers, suppressors, terminators, and the like, and that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences. On mRNA, a ribosome binding site is an expression control sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For

preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook *et al.*, *supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C. In a specific embodiment, "high stringency" refers to hybridization and/or washing conditions at 68°C in 0.2XSSC, at 42°C in 50% formamide, 4XSSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

A "vector" is a recombinant nucleic acid construct, such as plasmid, phage genome, virus genome, cosmid, or artificial chromosome to which another DNA segment may be attached. In a specific embodiment, the vector may bring about the replication of the attached segment, *e.g.*, in the case of a cloning vector. A segment of DNA is inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest and the segment and restriction sites are designed to ensure insertion of the segment in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change.

Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a Smurf1 or Smurf2 gene is heterologous to the plasmid vector DNA in which it is inserted for cloning or expression, and it is heterologous to a non-human host cell in which it is expressed, *e.g.*, a CHO cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

The invention provides antisense nucleic acids (including ribozymes), which may be used to inhibit expression of Smurf1, particularly to enhance the BMP pathway via Smads1 and 4. Thus, antisense nucleic acids corresponding to the Smurf1 gene, or a fragment thereof, can be used to alter BMP pathways. The invention also provides antisense nucleic acids to inhibit expression of Smurf2, to enhance the TGFβ signalling pathway. An "antisense nucleic acid" is a single stranded nucleic acid molecule which, upon hybridizing with complementary bases in an RNA or DNA molecule, inhibits the latter's role. If the RNA is a messenger RNA transcript, the antisense nucleic acid is a countertranscript or mRNA-interfering complementary nucleic acid. As presently used, "antisense" broadly includes RNA-RNA interactions, RNA-DNA interactions, ribozymes and RNase-H mediated arrest. Antisense nucleic acid molecules can be encoded by a

recombinant gene for expression in a cell (*e.g.*, U.S. Patent No. 5,814,500; U.S. Patent No. 5,811,234), or alternatively they can be prepared synthetically (*e.g.*, U.S. Patent No. 5,780,607).

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of Smurf1 or Smurf2, or to detect the presence of nucleic acids encoding Smurf1 or Smurf2. In a further embodiment, an oligonucleotide of the invention can form a triple helix with a Smurf1 or Smurf2 DNA molecule. In still another embodiment, a library of oligonucleotides arranged on a solid support, such as a silicon wafer or chip, can be used to detect various polymorphisms of interest. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

Specific examples of synthetic oligonucleotides envisioned for this invention include oligonucleotides that contain phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl, or cycloalkl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2, CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 and O-N(CH_3)-CH_2-CH_2 backbones (where phosphodiester is O-PO_2-O-CH_2). US Patent No. 5,677,437 describes heteroaromatic olignucleoside linkages. Nitrogen linkers or groups containing nitrogen can also be used to prepare oligonucleotide mimics (U.S. Patents No. 5,792,844 and No. 5,783,682). US Patent No. 5,637,684 describes phosphoramidate and phosphorothioamidate oligomeric compounds. Also envisioned are oligonucleotides having morpholino backbone structures (U.S. Pat. No. 5,034,506). In other embodiments, such as the peptide-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al.*, Science 254:1497, 1991). Other synthetic oligonucleotides may contain substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl,

substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-; S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2;NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substitued silyl; a fluorescein moiety; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Oligonucleotides may also have sugar mimetics such as cyclobutyls or other carbocyclics in place of the pentofuranosyl group. Nucleotide units having nucleosides other than adenosine, cytidine, guanosine, thymidine and uridine may be used, such as inosine.

As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the TGFβ superfamily) and homologous proteins from different species (*e.g.*, Smad (human), Mad (drosophila), etc.) (Reeck *et al.*, Cell 50:667, 1987). Such proteins, and their encoding genes, have sequence homology, as reflected by their high degree of sequence similarity.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck *et al.*, *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 70-75%, and most preferably at least about 80-85% of the nucleotides match over the defined length of the DNA sequences. An example of such a sequence is an allelic or species variant of a Smurf gene of the invention. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al.*, *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 70% of the amino acids are identical, or greater than about 90% are similar (functionally similar). Preferably, the similar or homologous sequences are identified by alignment using, for example, the

GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, BLAST, and Clustal W analysis (MacVector). Sequence comparison algorithms can also be found at http://www.nwfsc.noaa.gov/bioinformatics.html.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with

nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A nucleic acid coding a protein of the Smurf family, *e.g.*, Smurf1 or Smurf2, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human or xenopus cDNA or genomic library. Methods for obtaining genes are well known in the art, as described above (*see, e.g.*, Sambrook *et al.*, 1989, *supra*). In a specific embodiment, the invention provides cDNA sequences for human Smurf1 (hSmurf1) and Smurf2 (hSmurf2) genes **[SEQ ID NO: 1** and **SEQ ID NO: 3]**.

Accordingly, any animal cell can potentially serve as the nucleic acid source for the molecular cloning of a Smurf gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), including EST libraries and cDNA libraries prepared from tissues with high level expression of the protein (*e.g.*, a Xenopus Stage 9 (blastula) cDNA library–these are the cells that evidence the highest levels of expression of Smurf1). Other cell lines that may express Smurf1 or Smurf2 are frog blastula and gastula ectoderm, mesoderm, and endoderm; mouse embryonic stem cells; and various mammalian cells, such as NIH3T3, PC12, 293T, Hela, and COS. DNA encoding a Smurf protein can also be obtained by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook *et al.*, 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

Identification of a specific DNA fragment containing a desired Smurf gene can be accomplished in various ways known in the art. For example, a portion of a Smurf gene exemplified below can be purified and labeled to prepare a labeled probe, and the generated DNA may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, Science 196:180, 1977; Grunstein and Hogness, Proc. Natl. Acad. Sci. U.S.A. 72:3961, 1975). Those DNA fragments with substantial homology to the probe, such as an allelic variant from another individual, will hybridize. In a specific

embodiment, high stringency hybridization conditions are used to identify a homologous Smurf1 or Smurf2 gene.

The present invention also relates to cloning vectors containing genes encoding analogs and derivatives of a Smurf gene of the invention, *e.g.*, Smurf1 or Smurf2, that have similar or homologous functional activity. The production and use of derivatives and analogs related to Smurf1 and Smurf2 are within the scope of the present invention. For example, a truncated form of Smurf1 or Smurf2 can be provided. Such a truncated form includes Smurf1 or Smurf2 with a deletion. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type Smurf1 or Smurf2 of the invention. Such functions include binding to a Smad protein, *e.g.*, Smad1, Smad5 or Smad7, and catalyzing the ubiquitination of the bound Smad, of the activated TGFβ receptor/Smad complex, or another protein in a TGFβ/activin pathway. In another embodiment, the fragment has binding affinity but lacks or has reduced catalytic capacity.

A Smurf derivative can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Alternatively, non-functional mutant forms of the Smurf proteins, that may for example compete with the wild-type Smurf protein in the BMP pathway, but which are less effective in ubiquitination of Smads, can be prepared for use in treating disorders associated with compromised BMP signaling pathways as described above. In a specific embodiment, *infra*, the mutation is C710A, described further in the Examples. In another specific embodiment, with respect to Smurf 2, the mutation is C716A.

Further selection can be carried out on the basis of the properties of the gene, *e.g.*, if the gene encodes a protein product having the isoelectric, electrophoretic, amino acid composition, partial or complete amino acid sequence, antibody binding activity, or ligand binding profile of a Smurf protein as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, immunological, or functional properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isoelectric focusing or non-equilibrium pH gel electrophores is behavior, proteolytic digestion maps, or antigenic properties as determined for Smurf1, or Smurf2.

The present invention also relates to analogs, and derivatives of a Smurf protein, homologs from other species, and mutant variants, which have the same or a homologous functional activity. The production and use of derivatives, analogs, and mutant variants related to a Smurf protein are within the scope of this invention. In a

specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type Smurf1 or Smurf2 protein of the invention.

Smurf derivatives can be made by altering nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced functional activity relative to a native Smurf, *e.g.*, Smurf1 or Smurf2, or that lack a functional activity, such as catalytic activity in the Hect ubiquitin-ligase domain in the C-terminal portion of the Smurf proteins. In one specific embodiment, mutant hSmurf1 having a point mutant at C710A is provided which disrupts the catalytic activity of the Hect domain thereby precluding ubiquitination of Smads1 and 4. In another specific embodiment, mutant hSmurf2 having a point mutation at C716A is provided which disrupts the catalytic activity of the Hect domain, precluding proteolytic degradation of the TGFβ receptor-Smad 7 complex. Alternatively, Smurf protein derivatives may encode soluble fragments of a Smurf protein domain, *e.g.*, WW domain, that have the same or greater affinity for the natural ligands, e.g. Smads1, 5 or 7. Such soluble derivatives may be potent inhibitors of ligand (*i.e.*, Smads1, 5 or 7) binding to the Smurf proteins.

In another specific embodiment, derivatives or fragments of Smads1 and 4 can be made that bind Smurf1 and preclude the E3 ligase from further binding cellular Smad and preventing its ubiquitination. In another embodiment, derivatives or fragments of Smad 7 can be made that reduce the association of Smurf2 to both Smad 7 and the TGFβ receptor. Thus, in one embodiment the invention contemplates use of peptides containing the linker region of R-Smads having a PPXY sequence, which is a conserved motif recognized by WW domains (*see*, Rotin, Curr. Topics Microbiol. Immunol., 228:115-133, 1998 and (33)) such as those found in Smurf1 or Smurf2, and corresponding nucleic acid sequences.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a Smurf gene may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of Smurf genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, Smurf derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a Smurf protein, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino

acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point. Particularly preferred substitutions include:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free $CONH_2$ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces b-turns in the protein's structure.

The genes encoding Smurf derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned Smurf1 or Smurf2 gene sequence can be modified by any of numerous strategies known in the art (Sambrook *et al.*, 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of a Smurf, care should be taken to ensure that the modified gene remains within the same translational reading frame as the gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, a Smurf-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination

sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated Smurf gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., *et al.*, 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant *et al.*, 1986, Gene 44:177; Hutchinson *et al.*, 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB" linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pMal-c, pFLAG, pGBT9, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2μ plasmid.

Expression of Smurf Polypeptides

The nucleotide sequence coding for a Smurf protein, or antigenic fragment, derivative or analog thereof, or a functionally active derivative, including a chimeric protein, thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which

contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding a Smurf protein of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding a Smurf protein and/or its flanking regions.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. The host cell containing the recombinant vector comprising the nucleic acid encoding a Smurf protein of the invention is cultured in an appropriate cell culture medium under conditions that provide for expression by the cell. Useful host cells for expression of Smurf include C_2C_{12}, 293T, CHO, COS, HEK, Hela, HepG2, NIH3T3, PC12, P19 and other cell lines, and kidney, brain, and bone cells.

A recombinant Smurf protein of the invention, or functional fragment, derivative, chimeric construct, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook *et al.*, 1989, *supra*).

Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of a Smurf protein may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control Smurf gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous

sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, 1988, Gene 67:31-40), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage l, *e.g.*, NM989, and other phage DNA, e.g., M13 and

filamentous single stranded phage DNA; yeast plasmids such as the 2m plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences, and the like.

For example, in a baculovirus expression systems, both non-fusion transfer vectors, such as but not limited to pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*II, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlue*Bac*III (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991)). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-

LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and b-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and b-gal selection). and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express Smurf1. For example, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*H1, *Sac*I, *Kpn*1, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*H1, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Once a particular recombinant Smurf DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a nonglycosylated core protein product, while expression in yeast can produce a glycosylated product. Expression in eukaryotic cells can increase the likelihood of "native" folding of a heterologous mammalian protein. Moreover, expression in mammalian cells can provide a tool for reconstituting, or constituting, Smurf

activity. Furthermore, it is known in the art that different vector/host expression systems may affect processing reactions (*e.g.*, proteolytic cleavages) to a different extent.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu *et al.*, 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Analysis of Gene Expression Mediated by Smurf Functional Activity

In one embodiment, oligonucleotide array technology can be used, *e.g.*, to evaluate gene expression, after binding of Smurf1 to Smads1 or 5, or binding of Smurf2 to Smad7, and to identify gene expression that correlates with, or is distinct from, gene expression in TGFβ or BMP treated cells or cells of injured and/or healing tissue, tissue having cells in a tumorigenic state, and during cellular and developmental processes, such as mitosis, cell differentiation, embryonic pattern formation and development and organogenesis. For example, one could compare gene expression in TGFβ or BMP treated cells in the presence or absence of Smurf expression. GeneChip expression analysis (Affymetrix, Santa Clara, CA) generates data for the assessment of gene expression profiles and other biological assays. Oligonucleotide expression arrays simultaneously and quantitatively interrogate thousands of mRNA transcripts (genes or ESTs), simplifying large genomic studies. Each transcript can be represented on a probe array by multiple probe pairs to differentiate among closely related members of gene families. Each probe cell contains millions of copies of a specific oligonucleotide probe, permitting the accurate and sensitive detection of low-intensity mRNA hybridization patterns. Differential expression data can provide a clear understanding of cellular pathways.

After hybridization intensity data is captured, *e.g.*, using a Hewlett-Packard GeneArray™ scanner. Software can be used to automatically calculate intensity values for each probe cell. Probe cell intensities can be used to calculate an average intensity for each gene, which directly correlates with mRNA abundance levels. Expression data can be quickly sorted on any analysis parameter and displayed in a variety of graphical formats for any selected subset of genes. Standard and custom GeneChip expression probe arrays are available today for human, mouse, yeast and other organisms. The GeneChip product line will expand to include expression arrays for the analysis of additional organisms and application areas such as toxicology and pharmacogenomics.

Transgenic Vectors

Smurf can be introduced into cells to treat a disorder associated with excess BMP or TGFβ activation, such as cancer. Smurf activity can be evaluated by introducing a Smurf vector into a cell and monitoring the cell upon Smurf expression. This can be done *in vitro* or *in vivo*, or *in vitro* followed by transplantation *in vivo*, also termed *ex vivo*. Alternatively, as discussed above, Smurf or a Smurf inhibitor (antisense, ribozyme, or intracellular antibody) can be delivered by a vector in modulate Smads, *e.g.*, to prevent Smurf regulation of Smads where BMP or TGFβ activity is desired, such as in bone regeneration, or to study Smurf regulated processes *in vivo*.

Smurf activity can be inhibited by various means, including by delivery of a vector encoding a dominant-negative Smurf derivative (*e.g.*, a Cys710 to Ala mutant) to cells, by antisense nucleic acids (including ribozymes and triple-helix-forming oligonucleotides; these are described in detail *supra*), and by expression of anti-Smurf intracellular antibodies, *e.g.*, single chain Fv antibodies (see generally Chen, Mol. Med. Today, 3:160-167, 1997; Spitz *et al.*, Anticancer Res., 16:3415-3422, 1996; Indolfi *et al.*, Nat. Med., 2:634-635, 1996; Kijima *et al.*, Pharmacol. Ther., 68:247-267, 1995).

As discussed above, a vector is any means for the transfer of a nucleic acid according to the invention into a host cell. These include viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses and adeno-associated viruses. Thus, a gene encoding a functional or mutant Smurf protein or polypeptide domain fragment thereof can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [*see, e.g.*, Miller and Rosman, *BioTechniques* 7:980-990 (1992)]. Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication)

region. Such techniques may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsidating the viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt *et al.*, Molec. Cell. Neurosci., 2:320-330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 A], or other defective herpes virus vectors [International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994]; an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* [J. Clin. Invest., 90:626-630, 1992; *see also* La Salle *et al.*, Science, 259:988-990, 1993]; and a defective adeno-associated virus vector [Samulski *et al.*, J. Virol., 61:3096-3101, 1987; Samulski *et al.*, J. Virol., 63:3822-3828,1989; Lebkowski *et al.*, Mol. Cell. Biol., 8:3988-3996, 1988)].

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, *e.g.*, adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-g (IFN-g), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors [*see, e.g.*, Wilson, *Nature Medicine* (1995)]. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Adenovirus vectors. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard *et al.*, Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal

origin is a canine adenovirus, more preferably a CAV2 adenovirus (*e.g.* Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a specific embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero *et al.*, Gene 101:195 1991; EP 185 573; Graham, EMBO J. 3:2917, 1984). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the said adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the said elements, and (ii) contain the sequences which are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham *et al.*, J. Gen. Virol. 36:59 1977) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Adeno-associated viruses. The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions which carry the encapsidation functions: the left-hand part of the genome, which contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, which contains the cap gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the said gene of interest in vitro (into cultured cells) or in vivo, (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques.

Retrovirus vectors. In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, 1983, Cell 33:153; Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, 1988, J. Virol. 62:1120; Temin *et al.*, U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein *et al.* Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"),

HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

In general, in order to construct recombinant retroviruses containing a nucleic acid sequence, a plasmid is constructed which contains the LTRs, the encapsidation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions which are deficient in the plasmid. In general, the packaging cell lines are thus able to express the gag, pol and env genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (US4,861,719); the PsiCRIP cell line (WO90/02806) and the GP+envAm-12 cell line (WO89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences which may include a part of the gag gene (Bender *et al.*, J. Virol. 61 (1987) 1639). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retroviral vectors can be constructed to function as infections particles or to undergo a single round of transfection. In the former case, the virus is modified to retain all of its genes except for those responsible for oncogenic transformation properties, and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

Lentivirus vectors. In another embodiment, lentiviral vectors are can be used as agents for the direct delivery and sustained expression of a transgene in several tissue types, including brain, retina, muscle, liver and blood. The vectors can efficiently transduce dividing and nondividing cells in these tissues, and maintain long-term expression of the gene of interest.

Lentiviruses contain at least two regulatory genes, *tat* and *rev*, that are essential for replication, and four accessory genes that encode critical virulence factors. [For a review, see Naldini, L., Curr. Opin. Biotechnol., 9:457-63, 1998]. The viral sequences non-essential for transduction are eliminated thereby improving the biosafety of this particular vector. Self-inactivating HIV-1 vectors are known, which have a deletion in the 3' long terminal repeat (LTR) including the TATA box, and significantly improve the biosafety of HIV-derived vectors by reducing the likelihood that replication-competent retroviruses will originate in the vector producer and target cells (Zufferey, *et al.*, J. Virol., 72:9873-80, 1998). In addition, the deletion improves the potential performance of the vector by removing LTR sequences previously associated with transcriptional interference

and suppression in vivo and by allowing the construction of more-stringent tissue-specific or regulatable vectors.

Lentiviral packaging cell lines are available and known generally in the art. They facilitate the production of high-titer lentivirus vectors for gene therapy. An example is a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate virus particles at titers greater than 10(6) IU/ml for at least 3 to 4 days (Kafri, *et al.*, J. Virol., 73: 576-584, 1999). The vector produced by the inducible cell line can be concentrated as needed for efficiently transducing nondividing cells *in vitro* and *in vivo*.

Non-viral vectors. Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner, et. al., Proc. Natl. Acad. Sci. U.S.A., 84:7413-7417, 1987; *see* Mackey, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 85:8027-8031, 1988; Ulmer *et al.*, Science, 259:1745-1748, 1993]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, Science, 337:387-388, 1989]. Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting [*see* Mackey, et. al., *supra*]. Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931), peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931).

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a

DNA vector transporter [*see, e.g.*, Wu *et al.*, J. Biol. Chem., 267:963-967, 1992; Wu and Wu, J. Biol. Chem., 263:14621-14624, 1988; Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams *et al.*, Proc. Natl. Acad. Sci. USA, 88:2726-2730, 1991]. Receptor-mediated DNA delivery approaches can also be used [Curiel *et al.*, Hum. Gene Ther., 3:147-154, 1992; Wu and Wu, J. Biol. Chem., 262:4429-4432, 1987]. US Patent No. 5,580,859 discloses delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal.

Antibodies against Smurf Proteins

According to the invention, a Smurf polypeptide produced recombinantly or by chemical synthesis, and fragments, variants, or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize a Smurf polypeptide. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library. Such an antibody is specific for Smurf proteins; it may recognize a mutant form of a Smurf, or wild-type Smurf. These antibodies can be used to alter the BMP pathway by inhibiting a Smurf protein (*e.g.*, anti-Smurf intracellular antibodies) or for diagnostic purposes.

Various procedures known in the art may be used for the production of polyclonal antibodies to a Smurf polypeptide or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with a Smurf polypeptide, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, a Smurf polypeptide or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*Bacille Calmette-Guerin*) and *Corynebacterium parvum.*

For preparation of monoclonal antibodies directed toward a Smurf polypeptide, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (Nature 256:495-497, 1975), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today 4:72, 1983; Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in Monoclonal Antibodies and Cancer

Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals (International Patent Publication No. WO 89/12690, published 28 December 1989). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, J. Bacteriol. 159:870, 1984; Neuberger *et al.*, Nature 312:604-608, 1984; Takeda *et al.*, Nature 314:452-454, 1985) by splicing the genes from a mouse antibody molecule specific for a Smurf polypeptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

In accordance with the invention, techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778) can be adapted to produce a Smurf polypeptide-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a Smurf polypeptide, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Smurf activity can also be inhibited by expression of anti-Smurf intracellular antibodies, *e.g.*, single chain Fv antibodies, using techniques known in the art (see generally Chen, Mol. Med. Today 3:160-167, 1997; Spitz, *et al.*, Anticancer Res. 16:3415-3422, 1996; Indolfi *et al.*, Nat. Med. 2:634-635, 1996; Kijima *et al.*, Pharmacol. Ther. 68:247-267, 1995).

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and

immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of a Smurf polypeptide, one may assay generated hybridomas for a product which binds to a Smurf polypeptide fragment containing such epitope. For selection of an antibody specific to a Smurf polypeptide from a particular species of animal, one can select on the basis of positive binding with a Smurf polypeptide expressed by or isolated from cells of that species of animal.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of a Smurf polypeptide, *e.g.*, for Western blotting, imaging a Smurf polypeptide *in situ*, measuring levels thereof in appropriate physiological samples, etc., using any of the detection techniques mentioned above or known in the art. Such antibodies can also be used in assays for ligand binding, *e.g.*, as described in US Patent No. 5,679,582.

In a specific embodiment, antibodies that agonize or antagonize the activity of a Smurf polypeptide can be generated. Such antibodies can be tested using the assays described *infra* for identifying ligands.

Screening

According to the present invention, nucleotide sequences derived from the gene encoding a Smurf, and peptide sequences derived therefrom, are useful targets to identify drugs that are effective in treating disorders mediated by the BMP signaling pathway, as discussed above (*see*, Schmitt *et al.*, J. Orthopedic Res., 17:269, 1999) can be used to agonize or antagonize a Smurf protein. Drug targets include without limitation (i) isolated nucleic acids derived from the gene encoding a Smurf and (ii) isolated peptides and polypeptides derived from Smurf polypeptides.

In particular, identification and isolation of a Smurf protein provides for development of screening assays, particularly for high throughput screening of molecules that up- or down-regulate the activity of a Smurf, *e.g.*, by permitting expression of a Smurf protein in quantities greater than can be isolated from natural sources, or in indicator cells that are specially engineered to indicate the activity of a Smurf protein expressed from a gene after transfection or transformation of the cells. Accordingly, the present invention contemplates methods for identifying specific ligands of a Smurf protein using various screening assays known in the art.

Any screening technique known in the art can be used to screen for agonists or antagonists of a Smurf protein. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and agonize or antagonize a Smurf protein *in vivo*. For example, natural products libraries can be screened using assays of the invention for molecules that agonize or antagonize Smurf activity.

Knowledge of the primary sequence of Smurf1 and Smurf2, and the similarity of that sequence with proteins of known function, provides one having ordinary skill in the art with useful information to determine inhibitors or antagonists of a Smurf protein. Identification and screening of antagonists is further facilitated by determining structural features of the protein, *e.g.*, using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" (Scott and Smith, Science 249:386-390, 1990; Cwirla, *et al.*, Proc. Natl. Acad. Sci., 87:6378-6382, 1990; Devlin *et al.*, Science, 49:404-406, 1990), very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen *et al.*, Molecular Immunology 23:709-715, 1986; Geysen *et al.* J. Immunologic Method 102:259-274, 1987; and the method of Fodor *et al.* (Science 251:767-773, 1991) are examples. Furka *et al.* (14th International Congress of Biochemistry, Volume #5, Abstract FR:013, 1988; Furka, Int. J. Peptide Protein Res. 37:487-493, 1991), Houghton (U.S. Patent No. 4,631,211, issued December 1986) and Rutter *et al.* (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries (Needels *et al.*, Proc. Natl. Acad. Sci. USA 90:10700-4, 1993; Ohlmeyer *et al.*, Proc. Natl. Acad. Sci. USA 90:10922-10926, 1993; Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 9428028) and the like can be used to screen for Smurf ligands according to the present invention.

Test compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New

Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from *e.g.* Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle *et al.*, Tib Tech, 14:60, 1996).

In vitro *screening methods*

In one series of embodiments, an isolated nucleic acid comprising a Smurf gene is tested *in vitro* for its ability to bind test compounds in a sequence-specific manner. The methods comprise:

(i) providing a nucleic acid containing a particular sequence corresponding to all or portions of a Smurf protein;

(ii) contacting the nucleic acid with a multiplicity of test compounds under conditions appropriate for binding; and

(iii) identifying those compounds that bind selectively to the nucleic acid sequence.

Selective binding as used herein refers to any measurable difference in any parameter of binding, such as, *e.g.*, binding affinity, binding capacity, *etc.*

In one series of embodiments, an isolated peptide or polypeptide, or fragments thereof, comprising a Smurf protein is tested *in vitro* for its ability to bind test compounds in a sequence-specific manner. The screening methods involve:

(i) providing a peptide or polypeptide, or fragment thereof, corresponding to Smurf protein or a fragment thereof;

(ii) contacting the peptides with a multiplicity of test compounds under conditions appropriate for binding; and

(iii) identifying those compounds that bind selectively to the peptides.

In preferred embodiments, high-throughput screening protocols are used to survey a large number of test compounds for their ability to bind the genes or peptides disclosed above in a sequence-specific manner.

In vivo *screening methods*

Intact cells or whole animals expressing variants of a gene encoding a Smurf protein can be used in screening methods to identify candidate drugs. The following methods can be applied to normal or wild-type Smurf.

In one series of embodiments, a permanent cell line is established from an individual exhibiting expression of a variant Smurf gene. Alternatively, cells (including without limitation mammalian, mammalian, insect, yeast, and bacterial cells) are

programmed to express a gene comprising one or more variant Smurf sequences by introduction of suitable vector. Identification of candidate compounds can be achieved using any suitable assay, including without limitation (i) assays that measure selective binding of test compounds to particular variant of a Smurf; (ii) assays that measure the ability of a test compound to modify (*i.e.*, inhibit or enhance) a measurable activity or function of the Smurf; and (iii) assays that measure the ability of a compound to modify (*i.e.*, inhibit or enhance) the transcriptional activity of sequences derived from the promoter (*i.e.*, regulatory) regions of a Smurf gene.

In another series of embodiments, transgenic animals are created in which (i) a human Smurf, having one or more mutations is stably inserted into the genome of the transgenic animal; and/or (ii) endogenous Smurf genes are inactivated and replaced with a variant human Smurf gene. See, *e.g.*, Coffman, Semin. Nephrol. 17:404, 1997; Esther *et al.*, Lab. Invest. 74:953, 1996; Murakami *et al.*, Blood Press. Suppl. 2:36, 1996. A preferred method for creating such a transgenic animal is so called "knock-in" technology, where a human gene can be inserted to replace an endogenous gene under expression control of the endogenous genes' regulatory elements (*see*, Rodriguez *et al.*, Cell, 97:199, 1999). Such animals can be treated with candidate compounds and monitored for any alteration in the BMP and TGFβ/activin pathways.

Furthermore, populations that are not amenable to an established treatment for tissue and bone degeneration (*e.g.*, osteoporosis) or enhancing regeneration can be selected for testing of alternative treatments. Moreover, treatments that are not as effective in the general population, but that are highly effective in the selected population, may be identified that otherwise would be overlooked. This is an especially powerful advantage of the present invention, since it eliminates some of the randomness associated with clinical trials.

High-Throughput Screen

Agents according to the invention may be identified by screening in high-throughput assays, including without limitation cell-based or cell-free assays. It will be appreciated by those skilled in the art that different types of assays can be used to detect different types of agents. Several methods of automated assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period of time. Such high-throughput screening methods are particularly preferred. The use of high-throughput screening assays to test for agents is greatly facilitated by the availability of large amounts of purified polypeptides, as provided by the invention.

Reporter Genes

Smad proteins have been identified as mediators of intracellular signal transduction by members of the transforming growth factor-beta (TGF-beta) superfamily, which affect cell proliferation, differentiation, as well as pattern formation during early vertebrate development. Following receptor activation, Smads are assembled into heteromeric complexes consisting of a pathway-restricted Smad and the common Smad4 that are subsequently translocated into the nucleus where they are thought to play an important role in gene transcription. Smad Binding Elements (SBEs) containing the sequence CAGACA in the promoter of the *JunB* gene, is an immediate early gene that is potently induced by TGF-beta, activin, and bone morphogenetic protein (BMP) 2 (Jonk *et al.*, J. Biol. Chem., 273:21145, 1998). Two *JunB* SBEs are arranged as an inverted repeat, which is transactivated in response to Smad3 and Smad4 co-overexpression and shows inducible binding of a Smad3- and Smad4-containing complex in nuclear extracts from TGF-beta-treated cells. Smad proteins bind directly to the SBE. Multimerization of the SBE creates a powerful TGF-beta-inducible enhancer that is also responsive to activin and BMPs (*see, e.g.*, Kim *et al.*, Nature, 388:304, 1997).

Reporter gene expression can be tied to expression or activation of any component of the BMP signaling pathway. Any reporter gene known in the art can be used. Thus, nucleotide sequences having the SBE sequence can be isolated using techniques known in the art and operably linked to a reporter gene to determine whether a Smurf, derivatives, and variants thereof, alter the activity of such promoter. In addition, other known genes in the BMP signaling pathway, such as T1x2 or Smad7, which have promoters that are regulated by Smad1 and 5 can be operably linked to a reporter gene. In these assays, candidate compounds can be tested for their ability to alter BMP of TGFβ signaling in cells that express Smad1, Smad5, Smad7, Smurf1 or Smurf2, or derivatives or variants thereof.

Various reporter gene assays are known and can be used to evaluate the effects of a Smurf on a BMP or TGFβ/activin signaling pathway. Reporter genes include luciferase, β-galactosidase (β-gal or lac-Z), chloramphenicol acetyltransferase (CAT), horseradish peroxidase, and alkaline phosphatase. In addition, expression of almost any protein can be detected using a specific antibody. For example, a green fluorescent protein (GFP) expression assay permits evaluation of Smad-induced SBE activity. GFP has been modified to produce proteins that remain functional but have different fluorescent properties. Several U.S. patents teach use of GFP to visualize signaling pathways linked to the reporter gene by an inducible promoter. For example, see U.S. Patent No. 5,625,048 (modified GFP resulting in amino-acid changes provides visibly distinct colors and

increased intensities of emission), WO96/23898 (construct encoding a modified GFP which also contains an enzyme recognition site), WO97/11094 (fluorescent proteins with increased intensity), WO97/266333 (humanized GFP protein optimized to provide higher levels of expression in mammalian cells), WO97/42320 (modified GFP having increased intensity of fluorescence, WO98/06737 (modified GFP easily distinguished from green and blue fluorescent proteins), WO98/21355 (GFP mutants excitable using blue and white light).

Screening Kits

The components required to practice the screening methods described above can be prepared in kit form, for the convenience of the user. Such kits are preferably adapted for use in an automated screening apparatus.

Identification of Smurf1 Binding Partners

In still another embodiment, the present invention provides for identification of Smurf binding partners, in addition to Smad1, Smad7 and Smad5, which can then be analyzed for mutations that lead to diseases mediated by the BMP or TGFβ/activin pathways. One method for identifying such binding partners is a yeast two hybrid assay system, preferably using a hematopoietic stem cell library with yeast that are transformed with a recombinant Smurf. Alternatively, a Smurf protein can be used to affinity purify proteins from cell preparations, *e.g.*, using cells that endogenously produce Smurfs. Partially purified preparations can be probed with a labeled Smurf protein to identify specific binding partners, *e.g.*, in a Western-type or other antibody-assay type of system (see the description above of antibodies for examples of such assays; naturally, any protein can be labelled as an antibody and its binding to a binding partner evaluated).

EXAMPLES

The present invention may be better understood by reference to the following examples, which are not intended to limit the invention.

EXAMPLE 1

Smurf1 targets the BMP pathway and affects embryonic pattern formation. The TGFβ superfamily regulates diverse biological processes including cell growth, differentiation and pattern formation. Any misregulation of TGFβ signals may cause disease. Signals from activated TGFβ receptors are directly transduced from receptor to nucleus by Smad proteins. Currently, a few links have been established

between ubiquitin-mediated degradation and the modulation of morphogenetic signaling during development. This Example describes a new E3 ubiquitin ligase, Smurf1, that selectively interacts with BMP pathway-specific Smads to trigger their ubiquitination, degradation and loss of activity. In amphibian embryos, Smurf1 specifically blocked BMP signals and affected pattern formation. Thus, targeted ubiquitination of Smads may function to control both embryonic development and a wide variety of cellular responses to TGFβ signals.

Methods

Yeast two-hybrid screen. A Xenopus Smad1 cDNA (36) was cloned into the pGBT9 vector and used to screen a Xenopus oocyte cDNA library (Clonetech) by the yeast two-hybrid method (46) using Xenopus Smad1 as the bait protein. A partial cDNA was isolated and used to screen a Xenopus Stage9 (blastula) cDNA library to obtain a full length Smurf1 cDNA **[SEQ ID NO: 1]**. A human Smurf1 cDNA encoding all but the first 8 amino acids was identified in the EST database (AA292123), and was used to construct human Smurf1. The first 8 amino acids were reconstituted using the corresponding Xenopus Smurf1 cDNA sequence. hSmurf cDNA was FLAG tagged at its N-terminus. The ubiquitin ligase mutant version of hSmurf1 was generated by replacing cysteine 710 with alanine using a PCR-based approach and the mutation was confirmed by sequencing.

Co-immunoprecipitation. For immunoprecipitation assays Xenopus Smad1 (36), mouse Smad4, and human Smad2 (47), were FLAG-tagged at their C-termini and translated *in vitro* (rabbit reticulocyte extracts; Promega) in the presence of ^{35}S-Met. The FLAG-tagged Smads were bound to anti-FLAG antibody-conjugated beads (Kodak), washed in co-IP buffer (10mM Tris, pH 7.5, 90 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 10% glycerol, 1 mM phenylmethylsulfonylflouride) then incubated with ^{35}S-Met-labelled Smurf1 in the same buffer. After washing in co-IP buffer and elution in gel loading buffer, proteins were separated by SDS-PAGE and visualized by autoradiography.

Embryo methods and RT-PCR. Embryo production, preparation and injection of synthetic mRNA into embryos, animal cap and ventral marginal zone assays, embryonic RNA isolation, embryo fixation and whole mount *in situ* hybridization, and developmental RT-PCR were performed as described (36, 48). Primers for Smurf1 RT-PCR were 5'-GTCCTGTGACTGGAACCC-3' (sense) **[SEQ ID NO: 5]** and

5'-.GAGGACTGCTAGACAAT-3' (antisense) **[SEQ ID NO: 6]**, whose 5' ends are respectively located at positions 482 and 726 in the Smurf1 cDNA.

mSmurf1. Northern blots were performed of mSmurf1 expression in embryonic and adult mouse tissues. Equal amounts of PolyA+ mRNA from embryonic tissue 7, 11 and 15 days post coitum and tissue samples from testes, kidney, skeletal muscle, lung, spleen, brain and heart were analyzed. Cytoskeletal actin expression was assayed on the same blot and verified that mRNA loading was equal in all lanes. Whole-mount in situ hybridization on mouse embryos were also performed.

Immunoprecipitations and Immunoblotting. COS-1 and 293T cells were transiently transfected using lipofectAMINE (GibroBRL) and calcium phosphate precipitation methods, respectively (49). Immunoprecipitations and immunoblotting were performed as described previously (50) using anti-Flag M2 monoclonal antibody (Sigma), anti-Smad1/5 polyclonal antibody (50) or anti-WW2 Nedd4 (51) polyclonal antibody. Detection was achieved using the appropriate HRP conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham).

Pulse Chase Analysis. COS-1 cells were transfected as indicated above. Two days post-transfection the cells were labelled for 10 min. at 37° C with 50 μCi [^{35}S]-methionine/ml in methionine-free DMEM (Pulse). Cell layers were then washed two times and incubated in DMEM+10% FCS for the indicated time periods (Chase). At each time point of the chase, cell lysates prepared in TNTE lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 and 1mM EDTA) containing protease and phosphatase inhibitors were subjected to immunoprecipitations using an anti-Smad1 polyclonal antibody. Immune complexes were resolved by SDS-PAGE and visualized by autoradiography. A Phosphorimager (Molecular Dynamics) was used to quantitate the amount of metabolically labelled Smad1 present at each time point.

Ubiquitination Assay. 293T cells were transfected with HA-tagged ubiquitin, untagged-Smad1, and either Flag-hSmurf1 or Flag-hSmurf1 (C710A) as indicated above. Two days post-transfection, cells were lysed and subjected to a-Smad1 immunoprecipitation. The immunoprecipitates were then washed sequentially two times each in TNTE + 0.1% triton, SDS-RIPA (TNTE lysis buffer, 0.1% sodium dodecyl sulfate and 1% deoxycholate), and 500 mM LiCl, 50 mM Tris/HCl, pH 7.4 and 0.1% trition. The presence of HA-ubiquitinated Smad1 in the immune complexes was visualized by SDS-PAGE followed by immunoblotting with the monoclonal anti-HA 12CA5. Protein

levels of untagged Smad1, Flag-hSmurf1 and Flag-hSmurf1 (C710A) were analyzed by immunoblotting aliquots of total cell lysates with the appropriate antibodies.

Results

Isolation of an E3 ubiquitin ligase that interacts with the BMP signal transducer Smad1. To isolate factors that interact with and potentially regulate Smad1 we performed a yeast two hybrid screen using Xenopus Smad1 as the bait protein. Several overlapping cDNAs were isolated that encoded a protein with significant homology to the Hect subclass of E3 ubiquitin ligases (22). This novel gene and a closely related human homolog, Smurf1 and hSmurf1, respectively (for Smad ubiquitination regulatory factor-1), are proteins of 731 amino acids in length, share 91% sequence identity and contain a Hect ubiquitin-ligase domain in the C-terminal portion of the molecule. This domain is characteristic of a specific class of E3 ubiquitin ligases (22) that include in mammals, E6-AP and Nedd4 and in yeast, RSP5p and Pub1 (23-26).

Identification of human Smurf1 genomic location. The human Smurf1 genomic clone is located in PAC clone DJ0808A01, from chromosomal region 7q21.1-q31.1, located from nucleotides 2669..53763. A computer prediction of intron/exon splicing products generated a single conceptual mRNA with open reading frame directly corresponding to the actual cloned human cDNA.

E3 ubiquitin ligases function together with E1 and E2 enzymes to conjugate ubiquitin to specific protein substrates, which targets the protein for subsequent degradation by the proteasome (12). E1 enzymes recruit and activate ubiquitin, which is then transferred to E2, a ubiquitin conjugating enzyme. The E2 in turn attaches ubiquitin either directly to the protein to be modified, or transfers ubiquitin to the E3 ubiquitin ligase, which confers substrate selectivity to the ubiquitination complex. By functioning to recruit specific target proteins to the conjugation machinery, E3 activity provides selectivity to the ubiquitination process. E3 activity can be provided by structurally and functionally diverse proteins. For instance, E3s may exist as multimeric protein complexes that facilitate substrate recognition, but may or may not possess direct ligase activity. Examples of this include the N-end rule E3s, and the Skp1/Cullin/F-box (SCF) complexes (27-29). In the case of the Hect family of E3 ubiquitin ligases, of which Smurf1 is a member, a single protein provides both substrate specificity and catalytic activity (22, 30).

Smurf1 also displays several other structural features characteristic of RSP5, Nedd4 and Pub1 ubiquitin ligases. This includes an amino-terminal phospholipid and calcium binding C2 domain, as well as two WW domains, which facilitate protein-protein interactions by binding to PPXY motifs on partner proteins (31-33). Overall, Smurf1 is most closely related to Pub1 (**Fig. 1**), a ubiquitin ligase from *Sacromyces pombe* that regulates mitosis by targeting cdc25 for proteosomal degradation (23).

Expression of mSmurf1 in embryonic and adult tissue. Northern blots of mSmurf1 showed that a transcript of about 6 kb was the predominant form of Smurf1 expressed in embryos (**Fig. 2A**). In tissues, major transcripts of 3.0 and 6.0 kb were observed (**Fig. 2B**). Cytoskeletal actin expression was assayed on the same blot and verified that mRNA loading was equal in all lanes. In the mouse, mSmurf1 was present in very early development, from at least embryonic day 7 onward. In adults, mSmurf1 was expressed in most tissues assayed, but there were differences in the abundance of the transcripts. We did not observe detectable mSmurf1 transcripts in the skeletal muscle, and the kidney and spleen expressed low levels of the large transcript. The small transcript was very abundant in the testes, and it was very weakly or not at all expressed in the kidney, lung, spleen, brain and heart. Both blots were prepared by loading equal amounts of polyA+ RNA in each lane, but to account for any variation in RNA loading and transfer, we probed the blot with a tubulin probe, and found no substantial differences among lanes. Thus, the variation seen in the blots reflected the relative expression of mSmurf1 in embryos and tissues.

Whole-mount in situ hybridization on mouse embryos revealed Smurf1 expression in the nervous system, eye, branchial arches, axial mesoderm (notochord), somites, and limb buds from days p.c. 10 - 14.

Localization of Xenopus Smurf1 to the egg animal pole and embryonic ectoderm. In Xenopus embryonic development, Smurf1 mRNA was found expressed from egg through swimming tadpole stages, with maximum levels observed early in development at egg, blastula, and gastrula stages. Smurf1 levels declined sharply near the end of gastrulation, yet zygotic expression persisted at a reduced level into late swimming tadpole stages (**Fig. 3A**). Whole mount in situ hybridization (**Fig. 3B**) revealed that maternal Smurf1 transcripts were localized to the animal pole half of eggs and cleaving blastulae, which was confirmed by a northern blot analysis on RNA isolated from animal

and vegetal halves of middle blastula stage embryos (not shown). At gastrulation, Smurf1 expression became more widespread in the embryo, coincidently with the decline in its transcript levels observed by RT-PCR. During neural plate closure, however, Smurf1 expression was localized to the developing nervous system, and by tadpole stages it was highly expressed in the central nervous system, eye, pharyngeal pouches and somites. The embryonic expression pattern of Smurf1 partially overlaps with the expression of Smad1 and BMP-4 in the ectoderm at blastula and gastrula stages and the nervous system, eye, somites and pharyngeal pouches at tadpole stages (34-36).

Expression of hSmurf1 leads to selective reduction in the steady-state protein level of Smad1 and Smad5 in mammalian cells. Smurf1 contains a putative E3 ligase or Hect domain, and therefore was a candidate protein for interacting with Smad1. To investigate whether Smurf1 regulates steady-state levels of Smad1 protein, two mammalian cell lines, 293T and COS-1, were transfected with Smad1 alone or with increasing amounts of Flag-hSmurf1. The steady-state levels of Smad1 protein were then evaluated by immunoblots of whole cell lysates. Smad1 was readily detectable in the absence of hSmurf1 (**Fig. 4A**). However, expression of hSmurf1, even at low levels not readily detectable by Western blotting, produced a significant, dose-dependent decrease in Smad1 protein levels. At the highest levels of hSmurf1 expression there was no detectable Smad1 protein. Furthermore, co-expression of a constitutively activated form of the BMP type I receptor ALK6, which phosphorylates Smad1 (37, 38), did not alter hSmurf1-dependent decreases in Smad1 levels (**Fig. 4B**). Thus, expression of hSmurf1 causes dose-dependent decreases in steady-state levels of Smad1. This type of action can occur independently of activation of Smad1 by the type I BMP receptor.

To investigate whether Smurf1 activity is exclusive to Smad1, we investigated its effects on Smad2, a receptor-regulated Smad that functions in TGFβ and activin signaling pathways. Unlike Smad1, which was sensitive to the lowest doses of hSmurf1, there was only a slight effect on steady-state levels of Smad2 protein, which occurred only at the highest levels of Flag-hSmurf1 expression (**Fig. 4C**). Other Smads were tested: Flag-hSmurf1 had little or no effect on Smad3 or Smad4 protein levels, but it elicited a strong decrease in Smad5 protein, which is closely related to Smad1 (**Fig. 4D**). Together, these data demonstrate that hSmurf1 preferentially regulates the steady-state levels of Smad1 and Smad5, two receptor-regulated Smads that function in BMP signaling.

hSmurf1 regulates Smad1 degradation and ubiquitination. The inclusion of a putative E3 ligase or Hect domain supported the prediction that Smurf1 functions as an E3 ubiquitin ligase. To investigate whether Smurf1 regulates Smad degradation, studies focused on Smad1 (91% homology with Smad5). Analysis of Smad1 turnover by pulse-chase experiments revealed that in the absence of hSmurf1, Smad1 had a half life of approximately 6 hours. However, in the presence of hSmurf1, Smad1 turnover was significantly enhanced (half life of less than 2 hours) (**Fig. 5A**). Thus, hSmurf1 increases the rate of Smad1 turnover.

To determine the mechanism of hSmurf1-mediated turnover of Smad1, Smad1 ubiquitination in intact cells was assessed. To facilitate detection of ubiquitin, 293T cells were transfected with HA-tagged ubiquitin together with Smad1, in the presence or absence of Flag-hSmurf1. In the absence of hSmurf1, Smad1 displayed little or no detectable ubiquitination. However, upon co-transfection with hSmurf1 we observed the appearance of a ladder of ubiquitin-conjugated Smad1 (**Fig. 5B**). To confirm that ubiquitination of Smad1 required the catalytic activity of the Hect domain in hSmurf1, a point mutant in hSmurf1 (hSmurf1(C710A)) was constructed. This residue is critical for the catalytic activity of the Hect domain and the mutation is thought to target the cysteine residue that forms a thiolester bond with ubiquitin (22). In contrast to wildtype hSmurf1, expression of hSmurf1 (C710A) did not yield ubiquitinated Smad1. Moreover, hSmurf1 (C710A) did not affect Smad1 steady-state protein levels compared to wildtype hSmurf1, despite efficient expression of the mutant protein (**Fig. 5C**). Together, these data suggest that hSmurf1 alters Smad1 steady-state levels by inducing ubiquitin-mediated degradation of Smad1 via its Hect domain.

Smurf1 interacts selectively in vivo *with Smad1 and Smad5.* The interaction of Smurf1 with Smad proteins was investigated to assess the basis for the selective targeting of Smad1 and 5 for degradation by Smurf1. The yeast 2-hybrid assay was first used to test the ability of Xenopus Smurf1 to interact with Smad1, Smad4 or the non-specific control protein nuclear lamin. Yeast co-transfected with Smurf1 and Smad1 exhibited significant β-galactosidase activity; yeast co-transfected with Smurf1 and either Smad4 or lamin did not demonstrate β-galactosidase activity (**Fig. 6A**, left). Smurf1 selectively bound to Smad1, but not to Smad2 or Smad4 (**Fig. 6A**, right) as demonstrated by the capacity of ^{35}S-labelled Smurf1 to bind and immunoprecipitate Smads *in vitro.*

To test the capacity of ^{35}S-labelled Smurf1 to bind and immunoprecipitate Smads *in vitro* the specificity of Smurf1-Smad interactions in intact cells, the association of hSmurf1 with various Smads in 293T cells was investigated. Wild type hSmurf1 did not detect interactions with Smad1. However, it is possible that in intact cells Smurf1-Smad1 interactions are transient in nature, since demonstrating association between ubiquitin ligases and their substrates has proven difficult in other systems. By examining Smad1 interaction with the ubiquitin-ligase mutant Flag-hSmurf1 (C710A), association of the proteins could be detected (**Figs. 6B, C**). Furthermore, this interaction was unaffected by coexpression of the constitutively active BMP-type I receptor, ALK2 (data not shown), and is consistent with the notion that hSmurf1 regulates Smad1 turnover independent of BMP signaling. hSmurf1 (C710A) also bound efficiently to Smad5, but analysis of association with Smad2 revealed little, if any, interaction (**Figs. 6A** and **B**).

The linker regions of R-Smads contain a PPXY sequence, which is a conserved motif recognized by WW domains such as those found in Smurf1. Unlike wild type Smad1, a mutant of Smad1, in which the PY motif was deleted, associated only weakly with Smurf1 and was resistant to Smurf1-mediated degradation (data not shown). These results show that hSmurf1 associates specifically with Smad1 and Smad5, which interaction is mediated by binding between the PY motif in Smad1 and the WW domains of Smurf1.

The specificity of hSmurf1 as a ubiquitin ligase in targeting the Smads, was further tested by comparing it to Nedd4, a structurally related ubiquitin ligase. While Smad1 co-precipitated efficiently with hSmurf1 (C710A), it did not interact with the corresponding Nedd4 mutant. Consistent with this, overexpression of Nedd4 did not affect the steady-state level of Smad1 protein (**Fig. 6C**, lower panel). Together, these data indicate that both human and Xenopus Smurf1 proteins associate selectively with BMP-regulated Smad1 and Smad5, and that this interaction is specific to Smurf1 rather than a general feature of the Hect class of ubiquitin ligases.

Smurf1 antagonizes endogenous BMP signals in Xenopus embryos to dorsalize ventral mesoderm and neuralize ectoderm. In the Xenopus blastula, Smurf1 expression is localized to the animal pole ectoderm and partly overlaps the marginal zone, *i.e.*, a belt of cells located at the equator of the blastula which forms the mesoderm. This pattern of expression, considered together with the ability of Smurf1 to interact with, ubiquitinate, and degrade BMP pathway-specific Smads, suggests that Smurf1 may

function in ectodermal and mesodermal patterning by antagonizing BMP signals through Smad1 or Smad5.

Presently, naturally-occurring inhibitors of TGFβ activities have been described at the ligand level. Those factors, which include chordin, follistatin and noggin, act outside of cells by binding particular ligands, including BMPs and activin. During Xenopus mesoderm induction and patterning, BMP signals in the ventral part of the marginal zone specify development of tissues, such as blood and mesenchyme, that are characteristic of the ventral region of the tadpole. However, if BMP signaling is blocked by ligand antagonists such as chordin, follistatin, or noggin (which are secreted by the dorsal Spemann organizer), or by artificial inhibitors such as dominant negative BMP ligands or receptors, the prospective ventral mesoderm will differentiate into dorsal tissues such as muscle and notochord (a process referred to as "dorsalization") (5, 39).

Smurf1 has the potential to regulate BMP signals in the marginal zone, so ectopic Smurf1 expression was tested for interference with ventral tissue patterning. Smurf1 mRNA was microinjected into 4 cell blastula stage embryos in the ventral marginal zone (VMZ) to trigger the formation of ectopic secondary axial structures in the ventral region of 52% of tadpoles (n = 25). These secondary axial structures induced by Smurf1 were characteristic of dorsalization caused by BMP inhibition. Their formation was rescued by co-expression of Smad1 together with Smurf1, demonstrating that the effect of Smurf1 was limited to interference with the BMP/Smad1 pathway (**Fig. 7A**). Furthermore, overexpression of Smurf1 in cells of the dorsal marginal zone (DMZ), which forms head and dorsal axial structures, had no effect (data not shown). This latter observation was consistent with previous findings of the invention that Smurf1 does not target Smad2 in cultured cells.

The dorsalizing effects of Smurf1 using VMZ explants was further characterized. In this case, Smurf1 expression caused reduced blood differentiation and concomitant muscle differentiation, consistent with the dorsalizing effect of Smurf1 (**Fig. 7A**, right panel). As in the axis formation assay, co-expression of Smad1 with Smurf1 reversed these dorsalizing effects and demonstrated BMP pathway specificity.

In the ectodermal germ layer, endogenous BMP expression specifies epidermis, but when BMP signals are reduced or eliminated the ectoderm differentiates into cement gland or neural tissue, respectively (40, 41). The localization of Smurf1 mRNA in the ectoderm of the Xenopus blastula and early gastrula suggested that Smurf1

may regulate ectodermal patterning by modulating BMP signaling. Therefore, Smurf1 was tested for its presence in ectodermal tissue. It was found that overexpression of Smurf1 in animal caps triggers neural and cement gland differentiation, which is characteristic of a reduction in BMP signaling. These effects were reversed by co-expression of Smad1 in the animal cap (**Fig. 7B**). Together these results demonstrate that Smurf1 can block BMP signals in the ectoderm and mesoderm, suggesting that Smurf1 decreases BMP signaling in these tissues to affect embryonic patterning.

Smurf1 inhibits Smad1 activity but potentiates Smad2 activity in embryonic cells. In cultured cells, Smurf1 triggers ubiquitination and degradation of BMP pathway-specific Smads, and in Xenopus embryos Smurf1 antagonizes endogenous BMP signals. In Xenopus animal caps overexpression of Smads mimics the effects of TGFβ factors that signal through specific R-Smads. Thus, Smad1 induces exclusively ventral/posterior mesoderm, like BMP ligands, while Smad2 induces dorsal (Spemann organizer) mesoderm, like activin, Vg1 and nodal. We therefore tested whether Smurf1 can directly antagonize the mesoderm induction activities of Smad1 or Smad2 by overexpressing each Smad together with various doses of Smurf1 in animal caps. We found that expression of Smad1 alone (1 ng mRNA) induced ventral mesoderm, as demonstrated by expression of the ventral/posterior mesodermal markers Xhox3 and Xcad1. However, co-expression of Smurf1 and Smad1 blocked induction of these markers at all Smurf1 doses tested (**Fig. 8A**), demonstrating that Smurf1 can antagonize Smad1 activity.

To determine whether Smurf1 can interfere with Smad2, a dose of Smad2 (50 pg mRNA) was used in an amount sufficient to induce myoD (a vertebrate muscle marker of dorsal/lateral mesoderm), but insufficient to induce goosecoid (a frog marker for dorsal mesoderm of the Spemann Organizer) (Figs. 7B, C). When Smurf1 was co-expressed with Smad2 there was no inhibition of myoD induction at any of the Smurf1 doses tested (**Fig. 8B**). This was consistent with other findings that Smurf1 does not target Smad2. Interestingly, as the dose of Smurf1 was increased in the presence of this limiting amount (50 pg) of Smad2, induction of goosecoid gene expression was observed. This induction was dependent on Smad2 expression, since Smurf1 alone did not induce goosecoid. The response of cap cells to increasing amounts of Smurf1 in the presence of limiting Smad2 had similar effects to increasing the dose of Smad2 alone (**Fig. 8C**). Thus, a combination of 50 pg Smad2 and 100 pg Smurf1 induced goosecoid expression to a level

equivalent to a 5-fold higher (250 pg) dose of Smad2 alone (**Fig. 8C**). Thus, rather than inhibit Smad2 activity, Smurf1 appears to enhance the sensitivity of the animal cap to Smad2. These experiments demonstrate that in addition to blocking the response of animal pole cells to the BMP pathway, Smurf1 enhances these cells' response to the activin pathway. Although the invention is not dependent on any particular mechanism, the inventors propose that the Smurf1-mediated shift in responsiveness of animal pole cell results from lowered endogenous BMP signaling caused by targeted ubiquitination of the Smurf1 substrates, Smads 1 and 5. Therefore, Smurf1 functions during development to alter the competence of cells to respond to multiple TGFβ ligands by selective inactivation of a particular Smad pathway.

Discussion

The findings of the inventors provide a clear role, pathway and mechanism for selective ubiquitination in regulating developmental patterning. The Example presented evidence that TGFβ signaling can be controlled by ubiquitination of Smad signal transduction molecules, and that this activity has developmental consequences. Specifically, it was shown that Smurf1, a Hect family E3 ubiquitin ligase, selectively and directly interacts with BMP pathway-specific Smads, Smad1 and Smad5, triggering their ubiquitination and degradation. Smurf1 does not, however, interact with or affect TGFβ/activin pathway-specific Smad2, nor the common partner in Smad signal transduction complexes, Smad4. Furthermore, targeting of Smads by Hect ubiquitin ligases is not a general characteristic of E3 ligases since Nedd4 does not interact with Smads. Interestingly, the results showed Smurf1-mediated turnover of Smads is not affected by BMP signaling, suggesting that activated forms of Smad1 or Smad5 are not required to serve as Smurf1 substrates. Thus, Smurf1 does not act downstream of activated Smads to turn off BMP signals, but rather controls the competence of cells to respond to BMPs by regulating the steady state level of Smad protein in the cell.

The mSmurf1 gene is expressed in early development, where we predict it will regulate the response of cells to BMP signals. Smurf1 is present in adult tissues implying mSmurf1 regulates late stage BMP signals. Perhaps these signals act in tissue stasis, or perhaps ongoing differentiation associated with growth. To a large degree the expression in developing mouse at neurula stage and thereafter is in good accord with the expression patterns seen in the frog embryo at similar stages.

The phenotypic effects of Smurf1 in Xenopus embryos point to selective ubiquitination as an important regulator of inductive signals during embryonic development. Smurf1 dorsalizes mesoderm and neuralizes ectoderm by interfering with BMP signals that control patterning of these germ layers in normal development. Presently, regulation of BMP-dependent patterning is considered to be accomplished by secreted BMP-binding proteins, such as chordin, noggin and follistatin, which inhibit BMP signals extracellularly by direct binding to BMP ligands. However, Smurf1 provides a new mechanism to regulate BMP signals, which acts at the level of signal transduction, and presumably functions cell autonomously because Smurf1 is an intracellular protein. Furthermore, unlike secreted BMP binding proteins, which have restricted ligand specificity, Smurf1 provides broad inhibitory activity for BMP pathways because many type I receptors (ALK1, ALK2, ALK3, and ALK6) signal through Smad1 and Smad5 (42). Thus, in the developing embryo Smurf1 cooperates with extracellular BMP inhibitors to specify pattern formation. Whether Smurf1 is regulated in some manner remains an open question.

Of particular interest is the localization of Smurf1 mRNA to the animal pole of the Xenopus egg and blastula. This region forms ectodermal tissues such as epidermis, cement gland and the nervous system, tissue fates which are controlled by the level of BMP signaling perceived by the cells. When animal pole ectoderm is deprived of endogenous BMP signals neural differentiation occurs spontaneously, but as the levels of BMP ligand applied to cells, or the amount of Smad1 expressed within cells, are gradually increased, a spectrum of cell fates are progressively specified from neuronal, through cement glad then epidermis. Furthermore, reduced BMP signaling in the prospective ventral mesoderm of Xenopus embryos causes that tissue to be re-specified as dorsal mesoderm. Since Smurf1 can regulate cell responsiveness to BMP signals it may function in both ectodermal and mesodermal fate specificity by controlling the level of Smad1 or Smad5. High Smurf1 levels may completely shut off the ability of a cell to respond to BMPs, but at intermediate or low levels Smurf1 may modulate the magnitude of the BMP signal through the control of Smad1 protein levels to alter the nature of the cellular response. Consequently, Smurf1 may control cell fate determination in response to BMPs by establishing an intracellular morphogenetic gradient of Smad1 activity. In a variety of animals maternal mRNAs localized in eggs function as determinants of cell fate (43). In Xenopus, mRNAs encoding Vg1 (a TGFβ member) and Brat/VgT are localized to the egg

vegetal pole where they specify endoderm and mesoderm (44, 45). The results presented in this Example suggest that localized Smurf1 in the egg animal pole may function as a determinant of ectodermal cell fate.

An intriguing consequence of Smurf1 overexpression in Xenopus animal caps is altered cell competence to respond to Smad2, the activin/TGFβ pathway specific Smad. Smurf1 enhances the sensitivity of cells to a fixed level of ectopically expressed Smad2, so as Smurf1 levels increase, the mesoderm that is induced becomes progressively more characteristic of the Spemann organizer, the most dorsal type of mesoderm. This response mimics the effects of what happens when the levels of Smad2 alone are elevated in animal caps. Thus, Smurf1 can simultaneously change the competence of cells to respond to different TGFβ signals: it inhibits responses to the Smad1 pathway while stimulating responses to the Smad2 pathway. The distinct effects of Smurf1 on different TGFβ signaling pathways has important consequences for embryonic development, cell growth, tissue stasis and other biological functions regulated by the TGFβ superfamily.

EXAMPLE 2: Characterization and Cloning of human Smurf2

Human Smurf2 was identified and cloned using a Xenopus Smurf1 sequence in an EST database. Two overlapping EST clones corresponding to hSmurf2 were obtained and used to construct a full length sequence for hSmurf2. Smurf2 is closely related to Smurf1, displaying approximately 75% homology to the amino acid sequence of hSmurf1. Smurf2 contains a C2 domain at the amino-terminus, followed by three WW domains and a HECT ubiquitin ligase domain (**Fig. 12**). Smurf2 is closely related to Smurf1, but possesses an extra WW domain downstream of the C2 domain (**Fig. 13**). Smurf2 was found to be expressed throughout early development and was present in most adult tissues, with lower levels in spleen and skeletal muscle (**Figs.14A and 14B**). RT-PCR analysis further revealed that Smurf2 is expressed in a variety of cell lines that include P19, HepG2 and 293T.

Isolation of Human and mouse Smurf2. Several overlapping human clones displaying similarity to Smurf1 were identified from the expressed sequence tag (EST) database and a full-length version of Smurf2 was constructed by PCR using two overlapping EST clones. For northern blot analysis, a partial mouse Smurf2 cDNA clone encoding 225 amino acids of open reading frame including the stop codon and displaying

96% amino acid identity to human Smurf2 was identified in the EST database (I.M.A.G.E. clone ID 638876).

Construction of Plasmids. For mammalian expression constructs of Smurf2, the open reading frame was amplified by polymerase chain reaction (PCR) and was subcloned into pCMV5 in frame with an amino-terminal Flag or Myc tag (69). For Smurf2 WW domain deletions, amino acids 163-185 for DWW1, 257-279 for DWW2, and 303-325 for DWW3 were deleted. To generate the catalytically-inactive ubiquitin-ligase mutant of Smurf2, cysteine 716 was replaced with alanine. To generate the Smad7 PY mutants, tyrosine 211 was replaced with alanine (Y211A) or the PPPPY sequence between amino acid residues 206-212 was deleted (ΔPY). For TβRI-Flag, a Flag tag was introduced at the carboxy terminus of the receptor. All constructs were generated by PCR and confirmed by sequencing. The bacterial expression vectors, pET15-Smad7-HA and pGEX4T-1-Smurf2, were generated using convenient restriction sites.

Immunoprecipitation, Immunoblotting, and Affinity-labelling. For studies in mammalian cells, 293T and COS-1 cells were transiently transfected using calcium phosphate precipitation, or the DEAE-dextran method, respectively. Immunoprecipitation and immunoblotting were carried out using anti-HA monoclonal (12CA5, Boehringer), anti-HA rabbit polyclonal (Santa Cruz), anti-Myc monoclonal (9E10 ascites, Developmental Studies Hybridoma Bank), anti-Flag M2 monoclonal (Sigma) or anti-Smad7 rabbit polyclonal antibodies. For anti-Smad7 antibodies, rabbits were immunized with bacterially-produced GST-Smad7 encoding amino acids 202-260. After absorption of the antibody to either protein G or A–Sepharose, the precipitates were washed five times with TNTE 0.1% (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the appropriate antibody. Detection was conducted using the appropriate horseradish peroxidase (HRP)-conjugated sheep anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham). Bacterially-produced His-Smad7-HA was incubated with either Ni^{2+}-NTA beads (Qiagen) or with GST or GST-Smurf2-bound glutathione beads (Amersham), washed three times with TNTE (0.5% Triton X-100) and precipitates were analyzed by immunoblotting with anti-HA antibodies. For affinity-labelling, transfected COS-1 cells were incubated with 250 pM [^{125}I]TGF-b1 at 4 ^{0}C for 1 h, and receptors were cross-linked to ligand with DSS as described (70). The amount of

TβRI bound to Smurf2 or Smad7 was quantified by phosphorimaging (Molecular Dynamics).

Pulse-chase analysis and ubiquitination assay. COS-1 cells were transfected with the indicated constructs and two days post-transfection the cells were labelled for 15 min at 37 °C with 50 mCi/ml [^{35}S]-methionine (Trans [^{35}S]-label; Figure 2B) or 150 mCi/ml [^{35}S]-methionine (Figure 5C and D) in methionine-free Dulbecco's Modified Eagle's Medium (DMEM). Cell layers were then washed two times and incubated in DMEM containing 10% fetal bovine serum in the presence or absence of 30 mM lactacystin (obtained from E.J. Corey, Harvard University) or 0.4 mM chloroquine for the indicated times. At each time point, cell lysates were immunoprecipitated with anti-HA monoclonal antibodies, resolved by SDS-PAGE and visualized by autoradiography. Metabolically-labelled proteins were quantified by phosphorimaging. For the ubiquitination assay, 293T cells were transfected with HA-tagged ubiquitin and combinations of receptors, Smad7 and Smurf2. Cell lysates were subjected to anti-Smad7 immunoprecipitation and immunoprecipitates were boiled in 1% SDS for 5 min, diluted with TNTE 0.1% and reprecipitated with anti-Smad7 antibodies before anti-HA immunoblotting.

Subcellular localization by Immunofluorescence Deconvolution Microscopy. Mv1Lu cells, plated on gelatin-coated Permanox chamber slides (Nunc), were transfected with the indicated constructs by the calcium phosphate precipitation method. The cells were fixed, permeabilized, and reacted with the primary and secondary antibodies as described (69). Images were obtained using the Olympus 1X70 inverted microscope equipped with fluorescence optics and Deltavision deconvolution microscopy software (Applied Precision).

Transcriptional Response Assay. HepG2 cells were transiently transfected using the calcium phosphate DNA precipitation method with CMV-βgal, 3TP-Lux reporter construct and Smad7-HA constructs as indicated. Total DNA was kept constant by the addition of pCMV5 empty vector. The next day, cells were incubated overnight with or without 100 pM TGFβ. Luciferase activity was measured using the luciferase assay system (Promega) in a Berthold Lumat LB 96V luminometer and was normalized to β-galactosidase activity.

Results

Smurf2 does not regulate Smad steady-state levels. Smads were expressed in 293T cells in the absence or presence of Smurf2. Surprisingly, unlike Smurf1 which targets Smad1 for ubiquitin-mediated proteolysis, Smurf2 expression did not alter the steady-state levels of Smads 1, 2, 4 or 7 (**Fig. 15A**). To determine whether Smurf2 might associate with any of these Smads, the cell lysates were subjected to anti-Smurf2 immunoprecipitation and associated Smads examined by immunoblotting. In cells coexpressing Smad1, 2, or 4, no Smads were found to coprecipitate with Smurf2 under these conditions (**Fig. 15B**). In contrast, in cells expressing Smad7, a strong interaction between Smurf2 and Smad7 was detected. To confirm that Smurf2 expression did not alter Smad7 turnover, we also performed pulse-chase analysis of Smad7. Smad7 expressed in the absence or presence of Smurf2 displayed a similar half-life of approximately 4 h (**Fig. 15C**). To characterize the Smad7-Smurf2 association, GST-Smurf2 fusion protein was purified from bacteria and incubated with bacterially produced Smad7. Under these *in vitro* conditions Smad7 bound efficiently to Smurf2, indicating that Smurf2 associates directly with Smad7 (**Fig. 15D**). We also analyzed the determinants on Smad7 and Smurf2 that mediate their interaction. Smad7 possesses a PPXY sequence (PY motif) in its linker region, which can mediate interaction with WW domains such as those found in Smurf2 (71). The Smad7 PY motif was altered by changing the tyrosine residue to alanine (Smad7(Y211A)) or by deleting the entire motif (Smad7(ΔPY)). Analysis in 293T cells of Smurf2 binding to either Smad7 mutant revealed that the interaction with Smurf2 was reduced but not entirely abolished (**Fig. 15E**). This suggests that the PY motif makes an important contribution to the Smad7-Smurf2 interaction, however, it is not the sole determinant, and other regions in Smad7 may also contribute to their association. We also made mutants of Smurf2 in which each of the three WW domains were deleted. The interaction of Smad7 with a Smurf2 mutant that lacked the first WW domain was comparable to wild type Smurf2, however deletion of either WW2 or WW3 abolished any detectable interaction with Smad7 (**Fig. 15F**). Thus, the WW2 and WW3 domains in Smurf2 are both required to mediate binding to Smad7. Together, these results show that Smurf2 binds directly to Smad7 via its WW2 and WW3 domains but that it does not target Smad7 for ubiquitin-mediated proteolysis in the absence of TGFβ signalling.

Smad7 recruits Smurf2 into a complex with the TGFβ receptors. Smad7 binds heteromeric complexes of TGFβ type II (TβRII) and type I (TβRI) receptors through interactions with the activated type I receptor subunit (72, 56). The constitutive association between Smad7 and Smurf2 thus raised the interesting possibility that Smad7 might function to recruit Smurf2 to the TGFβ receptor complex. To test this, we expressed TGFβ receptors in COS-1 cells in the presence and absence of Smad7 and Smurf2. Receptors were then labelled by crosslinking to [^{125}I]-TGFβ. Affinity-labelled receptor complexes that co-precipitated with Smurf2 were visualized by autoradiography. In the absence or presence of Smad7, little or no TGFβ receptor complexes were found to co-precipitate with wild type Smurf2 (**Fig. 16**). A Smurf2 catalytic mutant was constructed in Smurf2 in which cysteine 716 was converted to an alanine residue (C716A). This mutation targeted the cysteine residue in the HECT ubiquitin-ligase domain that is thought to form a thiolester bond with ubiquitin. When Smurf2(C716A) was expressed alone we detected a slight interaction with the TGFβ receptors (**Fig. 16**). However in the presence of Smad7 the interaction of Smurf2(C716A) with the receptors was dramatically enhanced. Thus, Smad7 mediates the interaction of Smurf2 with the TGFβ receptors.

We also examined Smad7 bound to the receptors. Smad7 binds heteromeric TGFβ receptor complexes by interacting with the activated type I receptor subunit (72, 56). In the presence of wild type Smurf2 we observed a strong decrease in the amount of TGFβ receptor complexes that co-precipitated with Smad7 (**Fig. 16**, lane 3). This correlated with a decrease in the total amount of type I receptor present in these transfectants. Since Smad7 binds to receptor complexes via interactions with the activated type I receptor, these results suggest that Smurf2 decreases the levels of Smad7-bound receptor complexes. Consistent with this notion, when Smad7 was co-expressed with the catalytically inactive mutant of Smurf2, a decrease in Smad7-bound receptor levels was not observed (**Fig. 16**, lane 5). These results indicate that the catalytic activity of Smurf2 mediates the down-regulation of TGFβ receptors that are bound to Smad7.

Smad7 controls the subcellular localization of Smurf2. Smad7 resides in the nucleus and upon activation of TGFβ signalling is exported into the cytoplasm where it binds to TGFβ receptors through interaction with the receptor I subunit (73). To investigate whether Smad7 recruited Smurf2 to the receptors in intact cells, we determined whether Smad7 might regulate Smurf2 localization. For this, we expressed TβRII, TβRI

and Smurf2(C716A) in the absence or presence of Smad7 and examined the subcellular distribution of the appropriate protein by immunofluorescence microscopy.

Smad7 was localized to the nucleus, but in the presence of signalling was found predominantly in the cytoplasm, where it also colocalized with the transiently expressed TGFβ receptors in a punctate pattern. This distribution of TGFβ receptors has been observed previously by us and others (74-76). Smurf2 was similarly found predominantly in the nucleus, but this localization was not altered in the presence of TGFβ signalling. However, when Smad7 was coexpressed, the subcellular distribution of Smurf2 was dramatically altered and the protein was found predominantly outside of the nucleus and was extensively colocalized with the TGFβ receptors. These results suggest that Smad7 expression leads to export of Smurf2 from the nucleus and recruitment of Smurf2 to the TGFβ receptor complex.

Smurf2 induces degradation of TGFβ receptors and Smad7. The strong decrease in Smad7-associated receptors in the presence of wild type but not mutant Smurf2 suggested the possibility that Smurf2 might catalyze degradation of Smad7-bound receptor complexes. To analyze Smurf2-dependent turnover of TGFβ receptor complexes we coexpressed TßRII and TßRI in 293T cells. Under these conditions TßRII and TßRI assemble efficiently into functional heteromeric complexes, allowing us to investigate the turnover of the entire receptor pool. We first investigated steady-state levels of the receptors in cells expressing Smurf2 (**Fig. 17A**) and observed that Smurf2 had minimal effects on the steady-state levels of type II or type I receptors when the receptors were expressed either alone or together. However, in the presence of wild type Smad7, increasing Smurf2 expression led to a strong decrease in the steady-state levels of the type I receptor (**Fig. 17B**). In contrast, Smurf2(C716A) had no effect. Levels of type II receptor were also decreased upon expression of wild type Smurf2, although to a lesser extent than that observed for the type I receptor. This difference may be due to the fact that Smad7 binds receptor complexes via the activated type I receptor and does not interact with TßRII alone. We also tested whether Smurf2 targets a constitutively active version of the type I receptor, TßRI(T204D). This receptor mediates TGFβ signalling in the absence of the type II receptor and also binds Smad7 (72). Similar to receptor complexes, wild type Smurf2, but not Smurf2(C716A) caused a decrease in the steady-state levels of the activated type I receptor (**Fig. 17B**). To confirm that changes in receptor steady-state

levels reflected alterations in receptor turnover, we analyzed by pulse-chase analysis the half-life of TßRII and TßRI (**Fig. 17C**). Analysis of the type II receptor revealed that the newly synthesized protein had a half-life of approximately 1 h. In contrast TßRI was considerably more stable and displayed a half-life of approximately 4-6 h. Furthermore, the half-life of the type I receptor was unchanged when either Smad7 or Smurf2 were expressed individually with the receptors. However, when Smurf2 and Smad7 were co-expressed together with the TGFβ receptor complex, the half-life of the type I receptor was decreased to approximately 1 h. Thus, Smad7 and Smurf2 enhance the turnover of the type I receptor.

Ubiquitin-mediated proteolysis of membrane receptors can be mediated by both the proteasome and lysosome (60). To test whether Smurf2-dependent enhancement of TGFβ receptor degradation was dependent on proteasome and lysosome function we assessed the turnover of receptors in the presence and absence of lactacystin and chloroquine, which inhibit protein degradation by the proteasome and lysosome, respectively. Pulse-chase analysis of receptors revealed that each inhibitor on its own caused stabilization of a subset of the total TGFβ receptor pool (**Fig. 17D**). These results suggest that both the proteasome and the lysosome contribute to the enhanced turnover of the receptors that is observed in the presence of Smad7 and Smurf2.

In the course of these analyses of the TGFβ receptor we also evaluated Smad7 protein levels. In the absence of TGFβ receptor complexes Smad7 steady-state level and turnover was unaffected by Smurf2 (see **Fig. 15**). However, in the presence of TGFβ receptor complexes, Smad7 steady-state levels and half-life were decreased by Smurf2 expression (**Figs. 17B and 17C**, respectively). Furthermore, this decrease in Smad7 was dependent on the catalytic activity of the Smurf2 HECT domain, since expression of the Smurf2(C716A) mutant did not alter Smad7 levels. Smad7 turnover was also stabilized by lactacystin and chloroquine, suggesting that like the receptor complex, Smad7 is degraded by both proteasomal and lysosomal pathways. Thus, in the presence of TGFβ signalling Smurf2 induces degradation of Smad7, possibly by targeting the entire receptor-Smad7 complex.

To investigate ubiquitination of the receptors and Smad7, we expressed an HA epitope-tagged version of ubiquitin and evaluated ubiquitin-conjugates of TßRII, TßRI or Smad7 by immunoprecipitation followed by immunoblotting. To ensure specificity,

immunoprecipitated samples were boiled in SDS and then reprecipitated with the appropriate antibody prior to immunoblotting. Analysis of Smad7 ubiquitination revealed that in the absence or presence of Smurf2 there was little ubiquitin conjugated to the protein (**Fig. 17E**). However, when Smurf2 was coexpressed with Smad7 and the TGFβ receptors, we observed high molecular weight ubiquitin conjugates of Smad7 that were not detected when the catalytic mutant of Smurf2 was used. In contrast to Smad7, we were unable to detect significant Smurf2-dependent ubiquitination of the type I receptor. Since receptor turnover is increased in the presence of Smurf2 and Smad7, the inability to detect ubiquitin-conjugated receptors may reflect rapid degradation of the receptors that may occur once they are ubiquitinated. Alternatively, ubiquitination of Smad7 may serve as the signal that targets the entire receptor-Smad7 complex to the proteasome. Together these results show that Smad7-dependent recruitment of Smurf2 to the TGFβ receptor leads to proteasome and lysosome-mediated degradation of TGFβ receptor complexes and Smad7.

Association of Smurf2 enhances the inhibitory activity of Smad7. Our studies indicate that Smurf2 may be recruited to the TGFβ receptor complex via Smad7 and target the receptors for degradation. This suggests that ubiquitin-mediated degradation may contribute to the inhibitory activity of Smad7 in the TGFβ pathway. To test this we first investigated whether Smad7(Y211A), which displays poor interactions with Smurf2 (see **Fig. 15D**), has an altered ability to recruit Smurf2 to the TGFβ receptor. For this, wild type Smad7 or Smad7(Y211A) were coexpressed with TGFβ receptors in the presence or absence of Smurf2(C716A) and receptor interaction was measured by analyzing affinity labelled receptors that coprecipitated with either Smad7 or Smurf2. The interaction of TGFβ receptor complexes with Smad7(Y211A) was comparable to that observed with wild type Smad7 (**Fig. 18A**), demonstrating that this mutation in the PY motif does not affect Smad7 interaction with the TGFβ receptor. However, the ability of Smurf2(C716A) to associate with TGFβ receptors was substantially reduced in the presence of mutant Smad7, correlating with the reduced efficiency observed for the interaction between Smurf2 and Smad7(Y211A) described above. Next we investigated whether Smad7(Y211A) had an altered capacity to inhibit TGFβ signalling in HepG2 cells, which express endogenous Smurf2. For this, we assessed TGFβ signalling using the well characterized 3TP-lux reporter construct. Expression of wild type Smad7 strongly reduced TGFb-dependent induction of this reporter (**Fig. 18B**). In contrast, the

Smad7(Y211A) mutant had a substantially reduced inhibitory activity, despite its ability to interact efficiently with the TGFβ receptor complex. This suggests that binding of Smurf2 to Smad7 enhances Smad7 inhibitory activity towards the TGFβ signalling pathway.

Previous studies have shown that Smad7 binding to TGFβ receptor complexes prevents access of Smad2 to the receptors. Since Smad7(Y211A) interacts efficiently with the TGFβ receptor, we sought to determine whether the mutant protein might retain inhibitory activity to the TGFβ pathway at higher levels of expression. To examine this, we compared the relative efficiency of Smad7 versus Smad7(Y211A) in blocking TGFβ signalling by varying the amount of Smad7 expression. Wild type Smad7 potently inhibited TGFβ signalling at the lowest doses tested, whereas Smad7(Y211A) was much less efficient (**Fig. 18C**). However, at the highest dose tested, Smad7(Y211A) was capable of inhibiting TGFβ responsiveness. These results indicate that Smad7(Y211A) retains some inhibitory activity, probably through its ability to bind to the receptor and prevent access of Smad2 or Smad3. Together these data suggest that Smurf2 cooperates with Smad7 to promote its inhibitory activity.

Discussion

This Example confirms the identity of a new ubiquitin ligase, Smurf2, that functions in partnership with Smad7 to target the TGFβ receptor for degradation. Smurf2 bound TGFβ receptors very weakly and was unable to affect turnover of the receptors. However, in presence of Smad7, Smurf2 formed efficient complexes with the heteromeric TGFβ receptor and targeted the receptors for degradation. Since Smad7 binds directly to Smurf2 and also associates with the TGFβ receptor complex, these results indicated that Smad7 functions as an adaptor protein that mediates the interaction of Smurf2 with the TGFβ receptor complex (**Fig. 18D**). Consistent with this, mutations in the Smad7 PY motif that disrupted Smad7-Smurf2 interaction also interfered with the ability of Smad7 to recruit Smurf2 into a complex with the receptor. This mutant of Smad7 was also compromised in its ability to block TGFβ signalling, indicating that Smurf2 plays a role in mediating Smad7 inhibitory function. Since Smad7 competes with R-Smads for binding to the activated TGFβ receptor, this cooperation may be particularly important when Smad7 is expressed transiently or at low levels (53-58). By targeting receptors for degradation, Smurf2 can thus provide a mechanism for permanent inactivation of the Smad7-bound receptor complex. Concomitant with Smurf2-dependent degradation of

TGFβ receptors, we also observed degradation of Smad7 that was dependent on TGFβ signalling.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values are approximate, and are provided for description.

All patents, patent applications, publications, and other materials cited herein are hereby incorporated herein reference in their entireties.

References Cited

1. Hogan, B.L., Blessing, M., Winnier, G.E., Suzuki, N. & Jones, C.M. Growth factors in development: The role of TGF-β related polypeptide signaling molecules in embryogenesis. Development. (1995).

2. Kingsley, D.M. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes and Develop. 8, 133-146 (1994).

3. Roberts, A.B. & Sporn, M.B. The transforming growth factor-bs. Peptide Growth Factors and Their Receptors 1, 419-472 (1990).

4. Whitman, M. Smads and early developmental signaling by the TGFβ superfamily. Genes and Dev. 12, 2445-2462 (1998).

5. Harland, R. & Gerhart, J. Formation and Function of Spemann's organizer. Annu. Rev. Cell Biol. 13, 611-667 (1997).

6. Heldin, C.H., Miyazono, K. & ten Dijke, P. TGF-beta signaling from cell membrane to nucleus through SMAD proteins. Nature 390, 465-71 (1997).

7. Massague, J. TGF-beta signal transduction. Annu Rev Biochem 67, 753-791 (1998).

8. Wilson, P.A., Lagna, G., Suzuki, A. & Hemmati-Brivanlou, A. Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. Development 124, 3177-84 (1997).

9. Suzuki, A., Chang, C., Yingling, J.M., Wang, X.F. & Hemmati-Brivanlou, A. Smad5 induces ventral fates in Xenopus embryos. Dev. Biol. 184, 402-405 (1997).

10. Graff, J.M., Bansal, A. & Melton, D.A. Xenopus Mad proteins transduce distinct subsets of signals for the TGFβ superfamily. Cell 86, 1-20 (1996).

11. Baker, J.C. & Harland, R.M. A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. Genes Dev 10, 1880-9 (1996).

12. Hershko, A. & Ciechanover, A. The ubiquitin system. Ann. Rev. Biochem. 67, 425-479 (1998).

13. Hochstrasser, M. Ubiquitin-dependent protein degradation. Annu. Rev. Genet. , 405-439 (1996).

14. Pukatzki, S., Tordilla, N., Franke, J. & Kessin, R.H. A novel component involved in ubiquitination is required for development of Dictyostelium discoideum. J Biol Chem 273, 24131-8 (1998).

15. Lindsey, D.F., *et al.* A deubiquitinating enzyme that disassembles free polyubiquitin chains is required for development but not growth in Dictyostelium. J Biol Chem 273, 29178-87 (1998).

16. Chung, C.Y., Reddy, T.B., Zhou, K. & Firtel, R.A. A novel, putative MEK kinase controls developmental timing and spatial patterning in dictyostelium and is regulated by ubiquitin-mediated protein degradation [In Process Citation]. Genes Dev 12, 3564-78 (1998).

17. Epps, J.L. & Tanda, S. The Drosophila semushi mutation blocks nuclear import of Bicoid during embryogenesis. Curr. Biol. 8, 1277-1280 (1998).

18. Dickson, B.J. Photoreceptor development: breaking down the barriers. Curr Biol 8, R90-2 (1998).

19. Huang, Y., Baker, R.T. & Fischer-Vize, J.A. Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. Science 270, 1828-31 (1995).

20. Jiang, J. & Struhl, G. Regulation of the Hedgehog and Wingless signaling pathways by the F- box/WD40-repeat protein Slimb. Nature 391, 493-6 (1998).

21. Muralidhar, M.G. & Thomas, J.B. The Drosophila bendless gene encodes a neural protein related to ubiquitin-conjugating enzymes. Neuron 11, 253-66 (1993).

22. Huibregtse, J.M., Scheffner, M., Beaudenon, S. & Howley, P.M. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A 92, 2563-7 (1995).

23. Nefsky, B. & Beach, D. Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. EMBO J. 15, 1301-1312 (1996).

24. Scheffner, M., Huibregtse, J.M., Vierstra, R.D. & Howley, P.M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75, 495-505 (1993).

25. Hein, C., Springael, J., Volland, C., Haguenauer-Tsapis, R. & Andre, B. NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. 18, 77-87 (1995).

26. Kumar, S., *et al.* cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene. Genomics 40, 435-43 (1997).

27. Kwon, Y.T., *et al.* The mouse and human genes encoding the recognition component of the – end rule pathway. Proc Natl Acad Sci U S A 95, 7898-903 (1998).

28. Bartel, B., Wunning, I. & Varshavsky, A. The recognition component of the N-end rule pathway. Embo J 9, 3179-89 (1990).

29. Patton, E.E., Willems, A.R. & Tyers, M. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends. Genet. 14, 236-243 (1998).

30. Wang, G., Yang, J. & Huibregtse, J.M. Functional domains of the rsp5 ubiquitin-protein ligase. Mol Cell Biol 19, 342-52 (1999).

31. Nalefski, E.A. & Falke, J.J. The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. 5, 2375-2390 (1996).

32. Plant, P.J., Yeger, H., Staub, O., Howard, P. & Rotin, D. The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca2+- dependent plasma membrane localization. J
Biol Chem 272, 32329-36 (1997).

33. Staub, O. & Rotin, D. WW domains. Structure 4, 495-499 (1996).

34. Fainsod, A., Steinbeisser, H. & De Robertis, E.M. On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. Embo J 13, 5015-25 (1994).

35. Hemmati-Brivanlou, A. & Thomsen, G.H. Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. Dev Genet 17, 78-89 (1995).

36. Thomsen, G.H. Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. Development 122, 2359-66 (1996).

37. Hoodless, P.A., *et al.* MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85, 489-500 (1996).

38. Kretzschmar, M., Liu, F., Hata, A., Doody, J. & Massague, J. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev 11, 984-95 (1997).

39. Thomsen, G.H. Antagonism within and around the Spemann organizer: BMPs and their binding proteins in dorsal-ventral patterning. Trends Genet. 13, 209-211 (1997).

40. Sasai, Y. & De Robertis, E.M. Ectodermal patterning in vertebrate embryos. Dev Biol 182, 5-20 (1997).

41. Hemmati-Brivanlou, A. & Melton, D. Vertebrate embryonic cells will become nerve cells unless told otherwise. Cell 88, 13-17 (1997).

42. Kawabata, M., Imamura, T. & Miyazono, K. Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev 9, 49-61 (1998).

43. Bashirullah, A., Cooperstock, R.L. & Lipshitz, H.D. RNA localization in development. Annu Rev Biochem 67, 335-94 (1998).

44. Kimelman, D. & Griffin, K.J. Mesoderm induction: a postmodern view. Cell 94, 419-21 (1998).

45. Joseph, E.M. & Melton, D.A. Mutant Vg1 ligands disrupt endoderm and mesoderm formation in Xenopus embryos. Development 125, 2677-85 (1998).

46. Bartel, P. & Fields, S. Analyzing protein-protein interactions using two-hybrid system. Methods Enzymol. 254, 241-263 (1995).

47. Eppert, K., *et al.* MADR2 maps to 18q21 and encodes a TGFβ regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell 86, 543-552 (1996).

48. Horb, M.E. & Thomsen, G.H. A vegetally-localized Xenopus T-box gene specifies mesoderm and endoderm and is essential for mesoderm formation. Dev. 124, 1689-1698 (1997).

49. Wigler, M., *et al.* Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell 16, 777-85 (1979).

50. Macias-Silva, M., Hoodless, P.A., Tang, S.J., Buchwald, M. & Wrana, J.L. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273, 25628-36 (1998).

51. Staub, O., *et al.* Immunolocalization of the ubiquitin-protein ligase Nedd4 in tissues expressing the epithelial Na+ channel (ENaC). Am J Physiol 272, C1871-80 (1997).

52. Reddi, A.H., Role of morphogenetic proteins in skeletal tissue engineering and regeneration, Nature Biotechnology 16, 247-252 (1998).

53. Afrakhte, M., Morén, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. (1998). Induction of Inhibitory Smad6 and Smad7 mRNA by TGF-b Family Members. Biochem. Biophys. Res. Comm. *249*, 505-511.

54. Bitzer, M., von Gersdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A. A., Rojkind, M., and Bottinger, E. P. (2000). A mechanism of suppression of TGF-b/Smad signaling by NF-kB/RelA. Genes Dev. *14*, 187-197.

55. Ishiaki, A., Yamato, K., Nakao, A., Nonaka, K., Ohguchi, M., ten Dijke, P., and Nishihara, T. (1998). Smad7 Is an Activin-inducible Inhibitor of Activin-induced Growth Arrest and Apoptosis in Mouse B Cells. J. Biol. Chem. *273*, 24293-24296.

56. Nakao, A., Afrakhte, M., Morén, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997). Identification of Smad7, a TGFb-inducible antagonist of TGF-b signalling. Nature *389*, 631-635.

57. Takase, M., Imamura, T., Sampath, T. K., Takeda, K., Ichijo, H., Miyazono, K., and Kawabata, M. (1998). Induction of Smad6 mRNA by bone morphogenetic proteins. Biochem. Biophys. Res. Commun. *244*, 26-29.

58. Ulloa, L., Doody, J., and Massagué, J. (1999). Inhibition of transforming growth factor-b/SMAD signalling by the interferon-n/STAT pathway. Nature *397*, 710-713.

59. Bonifacino, J. S., and Weissman, A. M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Ann. Rev. Cell. Biol. *14*, 19-57.

60. Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol. *9*, 107-112.

61. Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999). Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor. EMBO J. *18*, 28-36.

62. van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (2000). Endocytosis and degradation of the Growth Hormone Receptor are proteasome-dependent. J. Biol. Chem. *275*, 1575-1580.

63. Harvey, K. F., and Kumar, S. (1999). Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. Trends Cell Biol. *9*, 166-169.

64. Staub, O., Abriel, H., Plant, P., Ishikawa, T., Kanelis, V., Saleki, R., Horisberger, J. D., Schild, L., and Rotin, D. (2000). Regulation of the epithelial Na+ channel by Nedd4 and ubiquitination. Kidney Int. *57*, 809-815.

65. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. EMBO J. *15*, 2371-2380.

66. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997). Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. EMBO J. *16*, 6325-6336.

67. Joazeiro, C. A. P., Wing, S. S., Huang, H.-K., Leverson, J. D., Hunter, T., and Liu, Y.-C. (1999). The tyrosine kinase negative regulator c-cbl as a RING-type E2-dependent ubiquitin-protein ligase. Science *286*, 309-312.

68. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y.

(1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol. Cell *4*, 1029-1040.

69. Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L., and Wrana, J. L. (1996). MADR1, a MAD-related protein that functions in BMP2 signalling pathways. Cell *85*, 489-500.

70. Macías-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996). MADR2 is a substrate of the TGFß receptor and its phosphorylation is required for nuclear accumulation and signalling. Cell *87*, 1215-1224.

71. Chen, H. I., and Sudol, M. (1995). The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. Proc Natl Acad Sci USA *82*, 7819-7823.

72. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y.-Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone Jr., M. A., Wrana, J. L., and Falb, D. (1997). The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell *89*, 1165-1173.

73. Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. (1998). Transforming Growth Factor b1 induces nuclear export of inhibitory Smad7. J. Biol. Chem. *273*, 29195-29201.

74. Gilboa, L., Wells, R. G., Lodish, H. F., and Henis, Y. I. (1998). Oligomeric structure of type I and type II Transforming Growth Factor-b receptors: Homodimers form in the ER and persist at the plasma membrane. J. Cell. Biol. *140*, 767-777.

75. Henis, Y. I., Moustakas, A., Lin, H. Y., and Lodish, H. F. (1994). The type II and III transforming growth factor-ß receptors form homo-oligomers. J. Cell Biol. *126*, 139-154.

76. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGF-b receptor. Cell *95*, 779-791.

77. Derynck, R., Zhang, Y., and Feng, X.-H. (1998). Smads: Transcriptional activators of TGF-b responses. Cell *95*, 737-740.

78. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997). TGF-b signalling from cell membrane to nucleus through SMAD proteins. Nature. *390*, 465-471.

79. Massagué, J., and Chen, Y. G. (2000). Controlling TGF-beta signaling. Genes Dev. *14*, 627-644.

80. Miyazono, K. (2000). TGF-beta signaling by Smad proteins. Cyto. Growth Factor Rev. *11*, 15-22.

81. Wrana, J. L. (2000). Regulation of Smad Activity. Cell *100*, 189-192.

82. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J.-I., Kawabata, M., and Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-b superfamily. Nature *389*, 622-626.

WHAT IS CLAIMED IS:

1. An isolated Smurf protein.

2. The Smurf protein of claim 1, which is human.

3. The Smurf protein of claim 1, which is a Smurf1.

4. The Smurf1 of claim 3 which has a mutation corresponding to C710A.

5. The Smurf1 of claim 3, which comprises at least 10 contiguous amino acid residues as depicted in a sequence of SEQ ID NO:2.

6. The Smurf1 of claim 3, which comprises an amino acid sequence as depicted in SEQ ID NO:2.

7. The Smurf protein of claim 1, which is a Smurf2.

8. The Smurf2 of claim 7 which has a mutation corresponding to C716A.

9. The Smurf2 protein of claim 7, which comprises at least 10 contiguous amino acid residues as depicted in SEQ ID NO:4.

10. The Smurf2 protein of claim 7, which comprises an amino acid sequence as depicted in SEQ ID NO:4.

11. An isolated nucleic acid encoding the Smurf protein of claim 1.

12. The nucleic acid of claim 11, wherein the Smurf protein is a human Smurf protein.

13. The nucleic acid of claim 11, wherein the Smurf protein is a Smurf1.

14. The nucleic acid of claim 13 which has a mutation corresponding to C710A.

15. The nucleic acid of claim 13, wherein the Smurf1 comprises at least 10 contiguous amino acid residues as depicted in a sequence of SEQ ID NO:2.

16. The nucleic acid of claim 13, wherein the Smurf1 comprises an amino acid sequence as depicted in SEQ ID NO:2.

17. The nucleic acid of claim 16, which has a nucleotide sequence as depicted in SEQ ID NO:1.

18. The nucleic acid of claim 11, wherein the Smurf protein is a Smurf2.

19. The nucleic acid of claim 18 which has a mutation corresponding to C716A.

20. The nucleic acid of claim 18, wherein the Smurf2 protein comprises at least 10 contiguous amino acid residues as depicted in SEQ ID NO:4.

21. The nucleic acid of claim 18, wherein the Smurf2 protein comprises an amino acid sequence as depicted in SEQ ID NO:4.

22. The nucleic acid of claim 18, which has a nucleotide sequence as depicted in SEQ ID NO:3.

23. A vector comprising the nucleic acid of claim 11.

24. A host cell comprising the vector of claim 23.

25. A method for producing a Smurf protein, comprising growing the host cell of claim 23 under conditions that permit expression of Smurf protein from the vector.

26. A method for producing a Smurf protein, comprising growing the host cell of claim 24 under conditions that permit expression of Smurf protein from the vector.

27. A transgenic non-human animal that expresses a human Smurf protein.

28. A method for inhibiting a bone morphogenic protein or tumor growth factor-beta activation pathway in a cell, which method comprises permitting the cell to grow under conditions that permit expression of Smurf from the vector of claim 23 introduced into the cell.

29. A method for inhibiting a bone morphogenic protein or tumor growth factor-beta activation pathway in a cell, which method comprises permitting the cell to grow under conditions that permit expression of Smurf from the vector of claim 24 introduced into the cell.

30. A method for promoting a bone morphogenic protein or tumor growth factor-beta activation pathway in a cell, which method comprises suppressing expression of endogenous Smurf in the cell.

31. A method of screening for a modulator of Smurf activity, which method comprises detecting modulation of Smurf activity in the presence of a test compound relative to Smurf activity in the absence of the test compound.

32. The method according to claim 31, wherein the Smurf activity is ubiquitination of a Smad protein in a host cell.

33. The method according to claim 31, wherein the Smurf activity is interaction of a Smurf WW domain with a PPXY domain of a Smad protein.

34. The method according to claim 33, wherein the test compound is screened for the ability to inhibit the interaction.

35. An antibody that specifically binds to Smurf protein.

36. An oligonucleotide or nucleic acid that specifically hybridizes under highly stringent conditions to an nucleic acid having a sequence encoding Smurf.

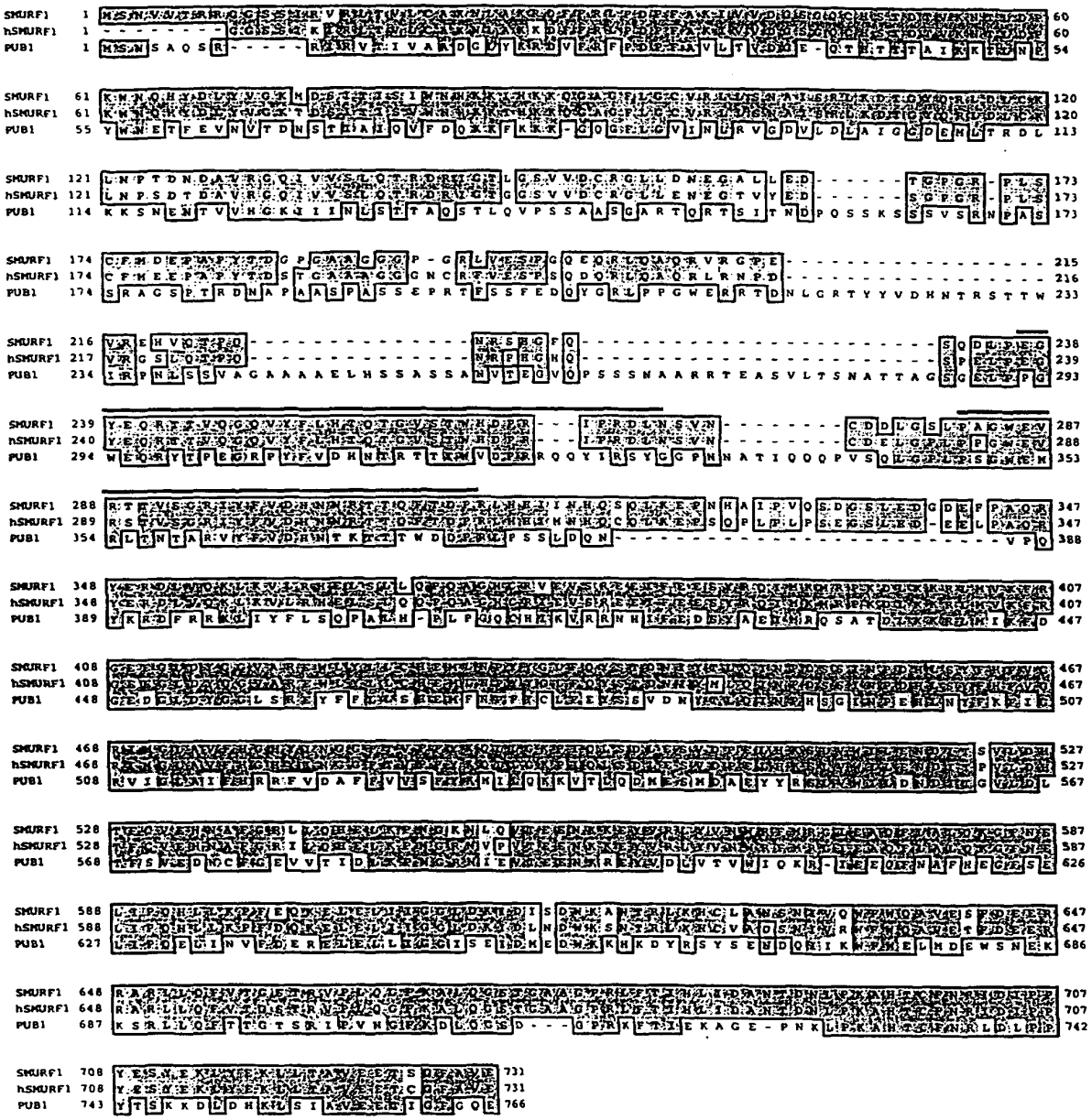

FIGURE 1

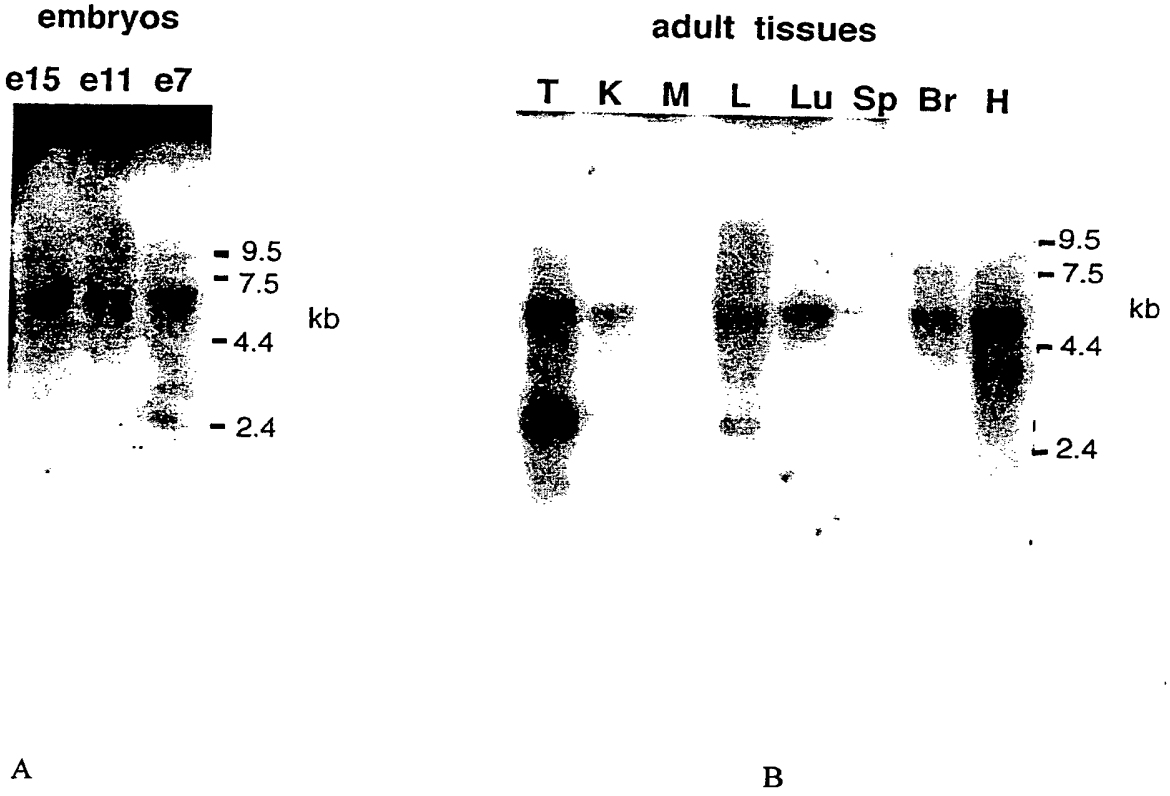
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FIGURE 2

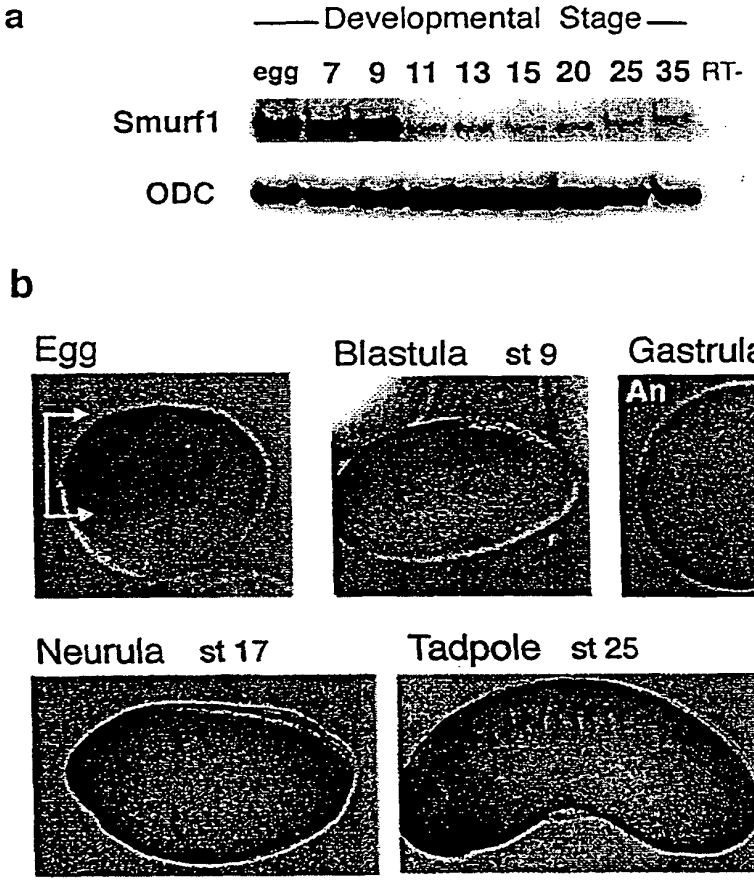
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FIGURE 3

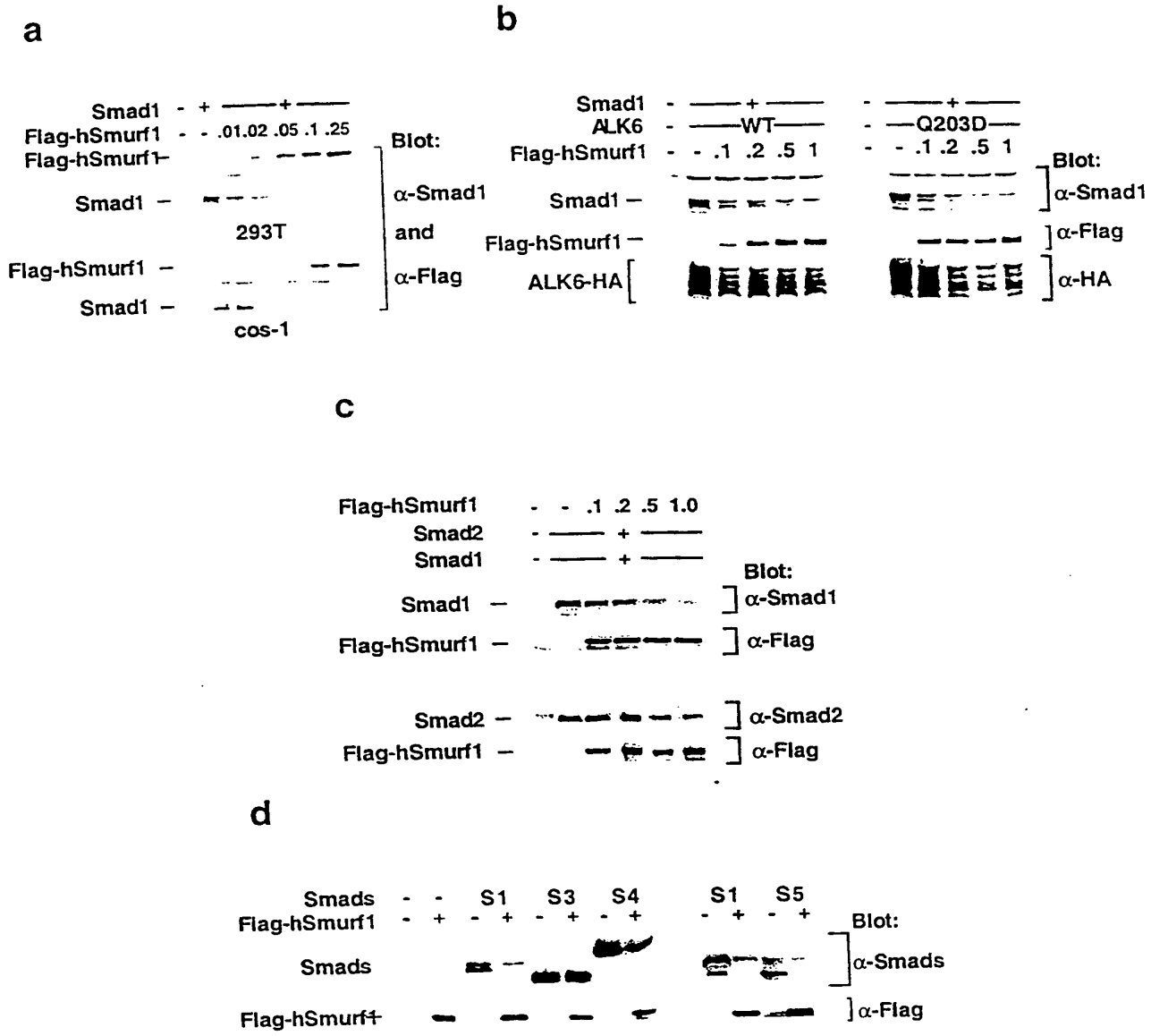

a
Smad1 - + ——+——
Flag-hSmurf1 - - .01.02 .05 .1 .25
Flag-hSmurf1 - — — —
Smad1 —
293T
Flag-hSmurf1 —
Smad1 —
cos-1
Blot:
α-Smad1
and
α-Flag
b
Smad1 - ——+—— - ——+——
ALK6 - ——WT—— - —Q203D—
Flag-hSmurf1 - - .1 .2 .5 1 - - .1 .2 .5 1
Smad1 —
Flag-hSmurf1 —
ALK6-HA
Blot:
α-Smad1
α-Flag
α-HA
c
Flag-hSmurf1 - - .1 .2 .5 1.0
Smad2 - —— + ——
Smad1 - —— + ——
Blot:
Smad1 — α-Smad1
Flag-hSmurf1 — α-Flag
Smad2 — α-Smad2
Flag-hSmurf1 — α-Flag
d
Smads - - S1 S3 S4 S1 S5
Flag-hSmurf1 - + - + - + - + - + - +
Blot:
Smads α-Smads
Flag-hSmurf1 α-Flag

FIGURE 4

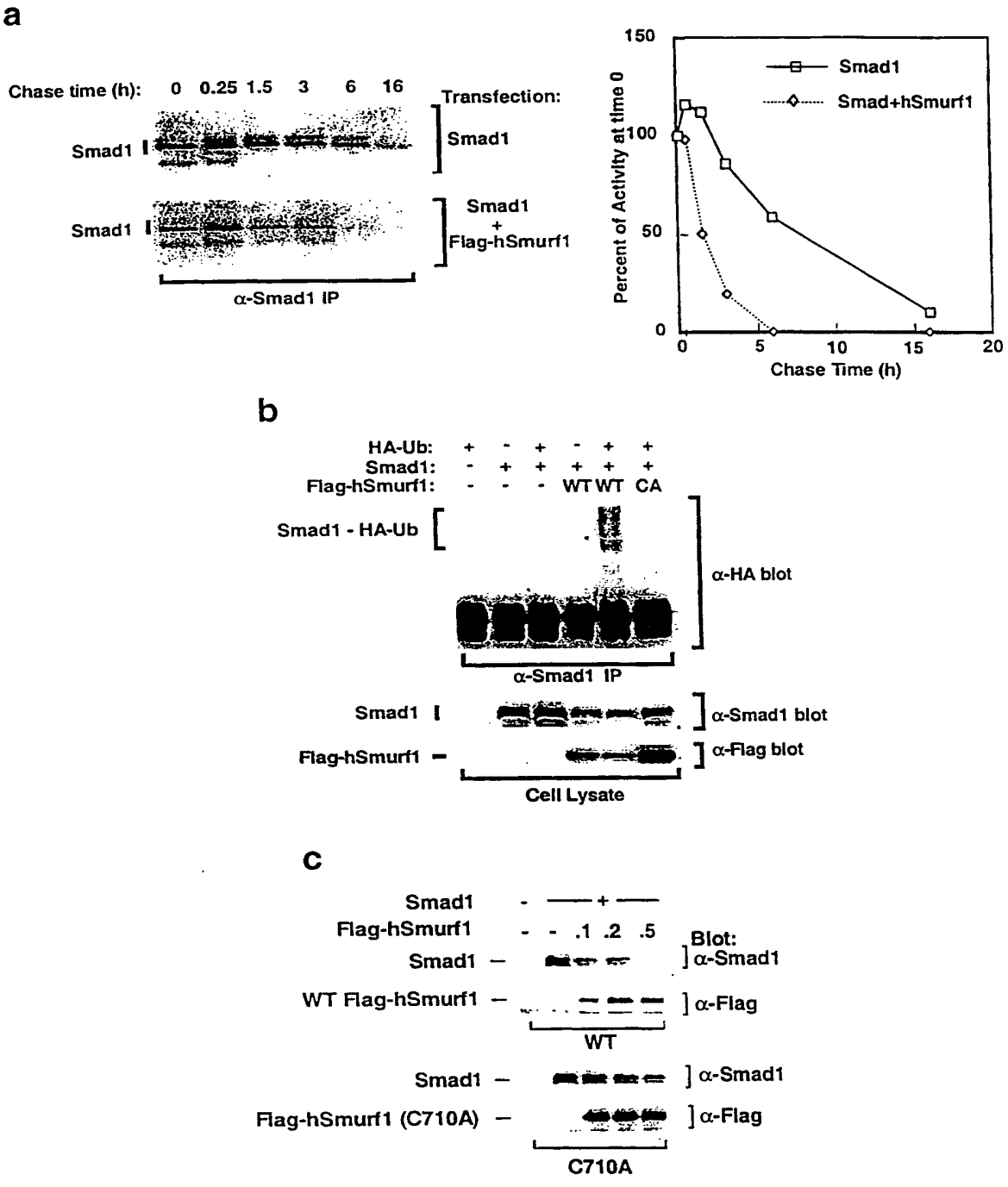

a
Chase time (h): 0 0.25 1.5 3 6 16
Transfection:
Smad1
Smad1
Smad1
Smad1
+
Flag-hSmurf1
α-Smad1 IP
150
100
50
0
Percent of Activity at time 0
Smad1
Smad+hSmurf1
0 5 10 15 20
Chase Time (h)
b
HA-Ub: + - + - + +
Smad1: - + + + + +
Flag-hSmurf1: - - - WT WT CA
Smad1 - HA-Ub
α-HA blot
α-Smad1 IP
Smad1
α-Smad1 blot
Flag-hSmurf1
α-Flag blot
Cell Lysate
c
Smad1 - —— + ——
Flag-hSmurf1 - - .1 .2 .5
Blot:
Smad1
α-Smad1
WT Flag-hSmurf1
α-Flag
WT
Smad1
α-Smad1
Flag-hSmurf1 (C710A)
α-Flag
C710A

FIGURE 5

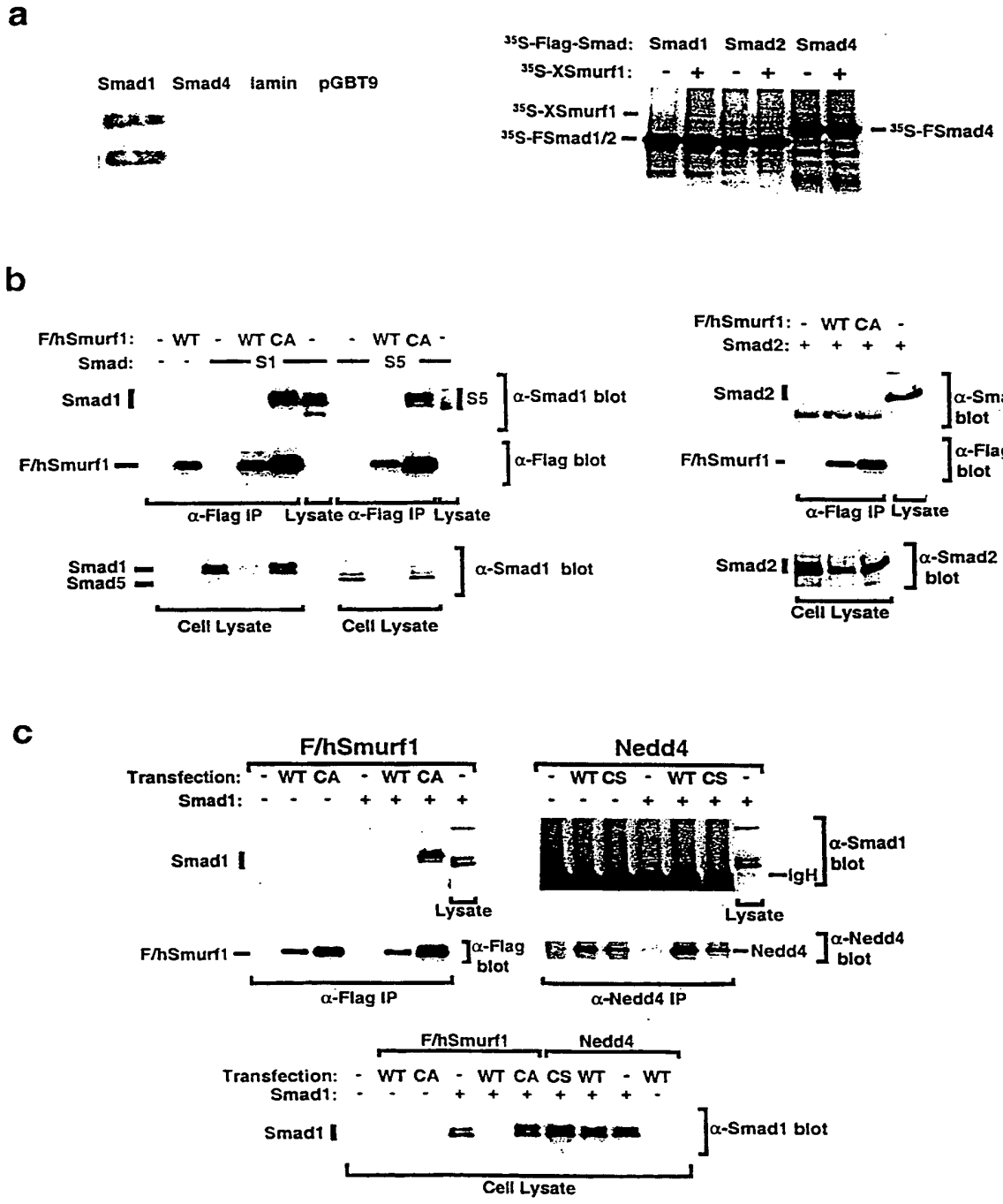
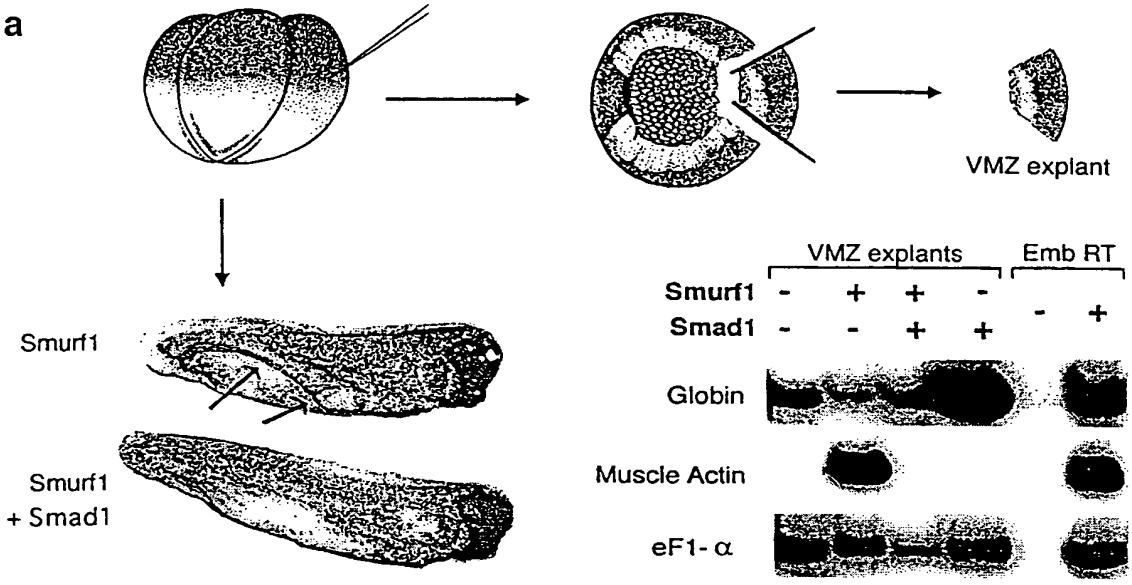

a
Smad1 Smad4 lamin pGBT9
35S-Flag-Smad: Smad1 Smad2 Smad4
35S-XSmurf1: - + - + - +
35S-XSmurf1
35S-FSmad1/2
35S-FSmad4
b
F/hSmurf1: - WT - WT CA - - WT CA -
Smad: - - S1 S5
Smad1
S5
α-Smad1 blot
F/hSmurf1
α-Flag blot
α-Flag IP Lysate α-Flag IP Lysate
Smad1
Smad5
α-Smad1 blot
Cell Lysate Cell Lysate
F/hSmurf1: - WT CA -
Smad2: + + + +
Smad2
α-Smad2 blot
F/hSmurf1
α-Flag blot
α-Flag IP Lysate
Smad2
α-Smad2 blot
Cell Lysate
c
F/hSmurf1
Nedd4
Transfection: - WT CA - WT CA - - WT CS - WT CS -
Smad1: - - - + + + + - - - + + + +
Smad1
α-Smad1 blot
IgH
Lysate
Lysate
F/hSmurf1
α-Flag blot
Nedd4
α-Nedd4 blot
α-Flag IP
α-Nedd4 IP
F/hSmurf1
Nedd4
Transfection: - WT CA - WT CA CS WT - WT
Smad1: - - - + + + + + + -
Smad1
α-Smad1 blot
Cell Lysate

FIGURE 6


a
VMZ explant
Smurf1
Smurf1
+ Smad1
VMZ explants
Emb RT
Smurf1 - + + -
Smad1 - - + +
- +
Globin
Muscle Actin
eF1-α

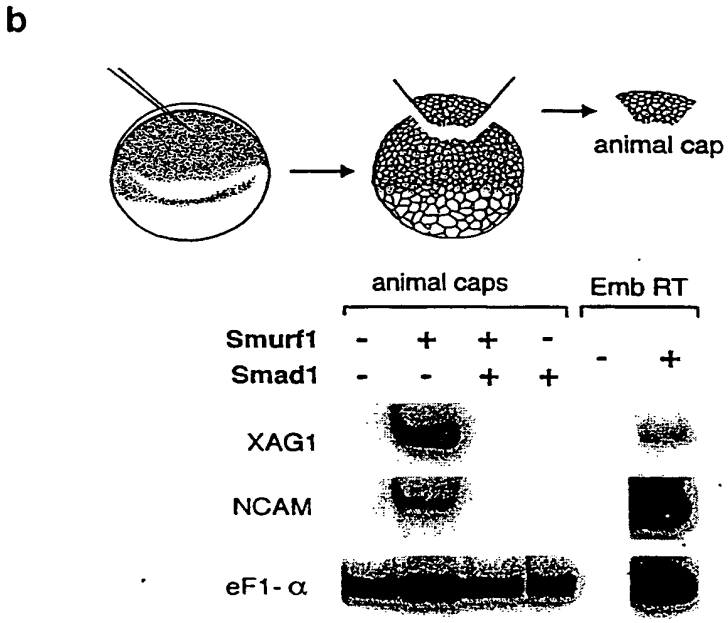
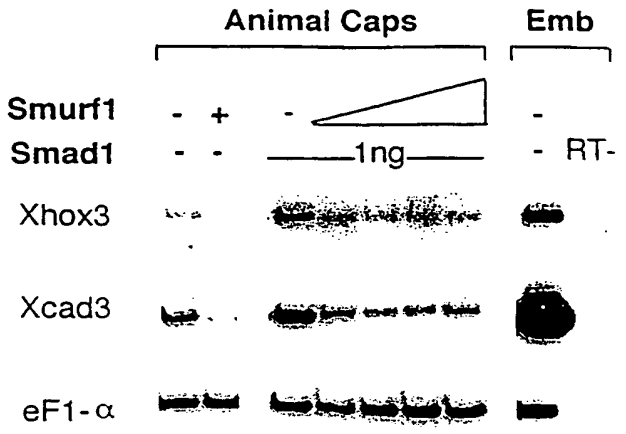
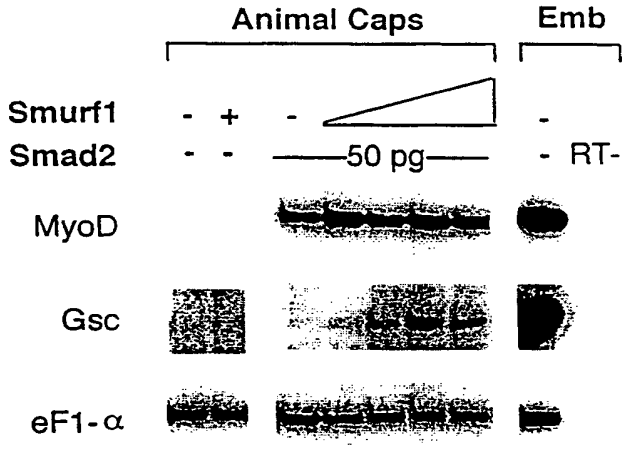

b
animal cap
animal caps
Emb RT
Smurf1 - + + -
Smad1 - - + +
- +
XAG1
NCAM
eF1-α

FIGURE 7

a


Animal Caps
Emb
Smurf1
Smad1
- + - - -
- - 1ng - RT-
Xhox3
Xcad3
eF1-α

b


Animal Caps
Emb
Smurf1
Smad2
- + - -
- - 50 pg - RT-
MyoD
Gsc
eF1-α

c

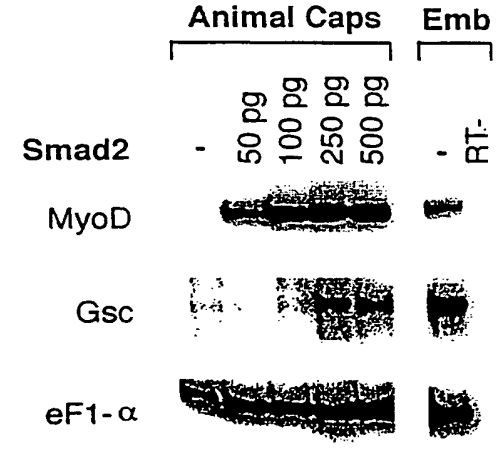

Animal Caps
Emb
Smad2
- 50 pg 100 pg 250 pg 500 pg - RT-
MyoD
Gsc
eF1-α

FIGURE 8

FIGURE 9

```
        10          20          30          40          50
         *           *           *           *           *
GGAGGCTCCA GCATCAAGAT CCGTCTGACA GTGTTATGTG CCAAGAACCT

        60          70          80          90          100
         *           *           *           *           *
TGCAAAGAAA GACTTCTTCA GGCTCCCTGA CCCTTTTGCA AAGATTGTCG

        110         120         130         140         150
         *           *           *           *           *
TGGATGGGTC TGGGCAGTGC CACTCAACCG ACACTGTGAA AAACACATTG

        160         170         180         190         200
         *           *           *           *           *
GACCCAAAGT GGAACCAGCA CTATGATCTA TATGTTGGGA AAACGGATTC

        210         220         230         240         250
         *           *           *           *           *
GATAACCATT AGCGTGTGGA ACCATAAGAA AATTCACAAG AAACAGGGAG

        260         270         280         290         300
         *           *           *           *           *
CTGGCTTCCT GGGCTGTGTG CGGCTGCTCT CCAATGCCAT CAGCAGATTA

        310         320         330         340         350
         *           *           *           *           *
AAAGATACCG GATACCAGCG TTTGGATCTA TGCAAACTAA ACCCCTCAGA

        360         370         380         390         400
         *           *           *           *           *
TACTGATGCA GTTCGTGGCC AGATAGTGGT CAGTTTACAG ACACGAGACA

        410         420         430         440         450
         *           *           *           *           *
GAATAGGAAC CGGCGGCTCG GTGGTGGACT GCAGAGGACT GTTAGAAAAT

        460         470         480         490         500
         *           *           *           *           *
GAAGGAACGG TGTATGAAGA CTCCGGGCCT GGGAGGCCGC TCAGCTGCTT

        510         520         530         540         550
         *           *           *           *           *
CATGGAGGAA CCAGCCCCTT ACACAGATAG CACCGGTGCT GCTGCTGGAG

        560         570         580         590         600
         *           *           *           *           *
GAGGGAATTG CAGGTTCGTG GAGTCCCCAA GTCAAGATCA AAGACTTCAG

        610         620         630         640         650
         *           *           *           *           *
GCACAGCGGC TTCGAAACCC TGATGTGCGA GGTTCACTAC AGACGCCCCA

        660         670         680         690         700
         *           *           *           *           *
GAACCGACCA CACGGCCACC AGTCCCCGGA ACTGCCCGAA GGCTACGAAC

        710         720         730         740         750
```

FIGURE 9

```
          *          *          *          *          *
AAAGAACAAC AGTCCAGGGC CAAGTTTACT TTTTGCATAC ACAGACTGGA

       760        770        780        790        800
          *          *          *          *          *
GTTAGCACGT GGCACGACCC CAGGATACCA AGAGACCTTA ACAGTGTGAA

       810        820        830        840        850
          *          *          *          *          *
CTGTGATGAA CTTGGACCAC TGCCGCCAGG CTGGGAAGTC AGAAGTACAG

       860        870        880        890        900
          *          *          *          *          *
TTTCTGGGAG GATATATTTT GTAGATCATA ATAACCGAAC AACCCAGTTT

       910        920        930        940        950
          *          *          *          *          *
ACAGACCCAA GGTTACACCA CATCATGAAT CACCAGTGCC AACTCAAGGA

       960        970        980        990       1000
          *          *          *          *          *
GCCCAGCCAG CCGCTGCCAC TGCCCAGTGA GGGCTCTCTG GAGGACGAGG

      1010       1020       1030       1040       1050
          *          *          *          *          *
AGCTTCCTGC CCAGAGATAC GAAAGAGATC TAGTCCAGAA GCTGAAAGTC

      1060       1070       1080       1090       1100
          *          *          *          *          *
CTCAGACACG AACTGTCGCT TCAGCAGCCC CAAGCTGGTC ATTGCCGCAT

      1110       1120       1130       1140       1150
          *          *          *          *          *
CGAAGTGTCC AGAGAAGAAA TCTTTGAGGA GTCTTACCGC CAGATAATGA

      1160       1170       1180       1190       1200
          *          *          *          *          *
AGATGCGACC GAAAGACTTG AAAAAACGGC TGATGGTGAA ATTCCGTGGG

      1210       1220       1230       1240       1250
          *          *          *          *          *
GAAGAAGGTT TGGATTACGG TGGTGTGGCC AGGGAGTGGC TTTACTTGCT

      1260       1270       1280       1290       1300
          *          *          *          *          *
GTGCCATGAA ATGCTGAATC CTTATTACGG GCTCTTCCAG TATTCTACGG

      1310       1320       1330       1340       1350
          *          *          *          *          *
ACAATATTTA CATGTTGCAA ATAAATCCGG ATTCTTCAAT CAACCCCGAC

      1360       1370       1380       1390       1400
          *          *          *          *          *
CACTTGTCTT ATTTCCACTT TGTGGGGCGG ATCATGGGGC TGGCTGTGTT

      1410       1420       1430       1440       1450
          *          *          *          *          *
```

FIGURE 9

```
CCATGGACAC TACATCAACG GGGGCTTCAC AGTGCCCTTC TACAAGCAGC

      1460        1470        1480        1490        1500
         *           *           *           *           *
TGCTGGGGAA GCCCATCCAG CTCTCAGATC TGGAATCTGT GGACCCAGAG

      1510        1520        1530        1540        1550
         *           *           *           *           *
CTGCATAAGA GCTTGGTGTG GATCCTAGAG AACGACATCA CGCCTGTACT

      1560        1570        1580        1590        1600
         *           *           *           *           *
GGACCACACC TTCTGCGTGG AACACAACGC CTTCGGGCGG ATCCTGCAGC

      1610        1620        1630        1640        1650
         *           *           *           *           *
ATGAACTGAA ACCCAATGGC AGAAATGTGC CAGTCACAGA GGAGAATAAG

      1660        1670        1680        1690        1700
         *           *           *           *           *
AAAGAATACG TCCGGTTGTA TGTAAACTGG AGGTTTATGA GAGGAATCGA

      1710        1720        1730        1740        1750
         *           *           *           *           *
AGCCCAGTTC TTAGCTCTGC AGAAGGGGTT CAATGAGCTC ATCCCTCAAC

      1760        1770        1780        1790        1800
         *           *           *           *           *
ATCTGCTGAA GCCTTTTGAC CAGAAGGAAC TGGAGCTGAT CATAGGCGGC

      1810        1820        1830        1840        1850
         *           *           *           *           *
CTGGATAAAA TAGACTTGAA CGACTGGAAG TCGAACACGC GGCTGAAGCA

      1860        1870        1880        1890        1900
         *           *           *           *           *
CTGTGTGGCC GACAGCAACA TCGTGCGGTG GTTCTGGCAA GCGGTGGAGA

      1910        1920        1930        1940        1950
         *           *           *           *           *
CGTTCGATGA AGAAAGGAGG GCCAGGCTCC TGCAGTTTGT GACTGGGTCC

      1960        1970        1980        1990        2000
         *           *           *           *           *
ACGCGAGTCC CGCTCCAAGG CTTCAAGGCT TTGCAAGGTT CTACAGGCGC

      2010        2020        2030        2040        2050
         *           *           *           *           *
GGCAGGGCCC CGGCTGTTCA CCATCCACCT GATAGACGCG AACACAGACA

      2060        2070        2080        2090        2100
         *           *           *           *           *
ACCTTCCGAA GGCCCATACC TGCTTTAACC GGATCGACAT TCCACCATAT

      2110        2120        2130        2140        2150
         *           *           *           *           *
GAGTCCTATG AGAAGCTCTA CGAGAAGCTG CTGACAGCCG TGGAGGAGAC
```

FIGURE 9

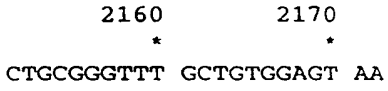


FIGURE 10

```
        10          20          30          40          50
         *           *           *           *           *
GGSSIKIRLT VLCAKNLAKK DFFRLPDPFA KIVVDGSGQC HSTDTVKNTL

        60          70          80          90          100
         *           *           *           *           *
DPKWNQHYDL YVGKTDSITI SVWNHKKIHK KQGAGFLGCV RLLSNAISRL

        110         120         130         140         150
         *           *           *           *           *
KDTGYQRLDL CKLNPSDTDA VRGQIVVSLQ TRDRIGTGGS VVDCRGLLEN

        160         170         180         190         200
         *           *           *           *           *
EGTVYEDSGP GRPLSCFMEE PAPYTDSTGA AAGGGNCRFV ESPSQDQRLQ

        210         220         230         240         250
         *           *           *           *           *
AQRLRNPDVR GSLQTPQNRP HGHQSPELPE GYEQRTTVQG QVYFLHTQTG

        260         270         280         290         300
         *           *           *           *           *
VSTWHDPRIP RDLNSVNCDE LGPLPPGWEV RSTVSGRIYF VDHNNRTTQF

        310         320         330         340         350
         *           *           *           *           *
TDPRLHHIMN HQCQLKEPSQ PLPLPSEGSL EDEELPAQRY ERDLVQKLKV

        360         370         380         390         400
         *           *           *           *           *
LRHELSLQQP QAGHCRIEVS REEIFEESYR QIMKMRPKDL KKRLMVKFRG

        410         420         430         440         450
         *           *           *           *           *
EEGLDYGGVA REWLYLLCHE MLNPYYGLFQ YSTDNIYMLQ INPDSSINPD

        460         470         480         490         500
         *           *           *           *           *
HLSYFHFVGR IMGLAVFHGH YINGGFTVPF YKQLLGKPIQ LSDLESVDPE

        510         520         530         540         550
         *           *           *           *           *
LHKSLVWILE NDITPVLDHT FCVEHNAFGR ILQHELKPNG RNVPVTEENK

        560         570         580         590         600
         *           *           *           *           *
KEYVRLYVNW RFMRGIEAQF LALQKGFNEL IPQHLLKPFD QKELELIIGG

        610         620         630         640         650
         *           *           *           *           *
LDKIDLNDWK SNTRLKHCVA DSNIVRWFWQ AVETFDEERR ARLLQFVTGS

        660         670         680         690         700
         *           *           *           *           *
TRVPLQGFKA LQGSTGAAGP RLFTIHLIDA NTDNLPKAHT CFNRIDIPPY

        710         720
```

FIGURE 10

```
                    *           *
ESYEKLYEKL LTAVEETCGF AVE*
```

FIGURE 11

```
        10          20          30          40          50
         *           *           *           *           *
ATGTCTAACC CCGGACGCCG GAGGAACGGG CCCGTCAAGC TGCGCCTGAC

        60          70          80          90          100
         *           *           *           *           *
AGTACTCTGT GCAAAAAACC TGGTGAAAAA GGATTTTTTC CGACTTCCTG

        110         120         130         140         150
         *           *           *           *           *
ATCCATTTGC TAAGGTGGTG GTTGATGGAT CTGGGCAATG CCATTCTACA

        160         170         180         190         200
         *           *           *           *           *
GATACTGTGA AGAATACGCT TGATCCAAAG TGGAATCAGC ATTATGACCT

        210         220         230         240         250
         *           *           *           *           *
GTATATTGGA AAGTCTGATT CAGTTACGAT CAGTGTATGG AATCACAAGA

        260         270         280         290         300
         *           *           *           *           *
AGATCCATAA GAAACAAGGT GCTGGATTTC TCGGTTGTGT TCGTCTTCTT

        310         320         330         340         350
         *           *           *           *           *
TCCAATGCCA TCAACCGCCT CAAAGACACT GGTTATCAGA GGTTGGATTT

        360         370         380         390         400
         *           *           *           *           *
ATGCAAACTC GGGCCAAATG ACAATGATAC AGTTAGAGGA CAGATAGTAG

        410         420         430         440         450
         *           *           *           *           *
TAAGTCTTCA GTCCAGAGAC CGAATAGGCA CAGGAGGACA AGTTGTGGAC

        460         470         480         490         500
         *           *           *           *           *
TGCAGTCGTT TATTTGATAA CGATTTACCA GACGGCTGGG AAGAAAGGAG

        510         520         530         540         550
         *           *           *           *           *
AACCGCCTCT GGAAGAATCC AGTATCTAAA CCATATAACA AGAACTACGC

        560         570         580         590         600
         *           *           *           *           *
AATGGGAGCG CCCAACACGA CCGGCATCCG AATATTCTAG CCCTGGCAGA

        610         620         630         640         650
         *           *           *           *           *
CCTCTTAGCT GCTTTGTTGA TGAGAACACT CCAATTAGTG GAACAAATGG

        660         670         680         690         700
         *           *           *           *           *
TGCAACATGT GGACAGTCTT CAGATCCCAG GCTGGCAGAG AGGAGAGTCA

        710         720         730         740         750
```

FIGURE 11

```
          *          *          *          *          *
GGTCACAACG ACATAGAAAT TACATGAGCA GAACACATTT ACATACTCCT

       760        770        780        790        800
          *          *          *          *          *
CCAGACCTAC CAGAAGGCTA TGAACAGAGG ACAACGCAAC AAGGCCAGGT

       810        820        830        840        850
          *          *          *          *          *
GTATTTCTTA CATACACAGA CTGGTGTGAG CACATGGCAT GATCCAAGAG

       860        870        880        890        900
          *          *          *          *          *
TGCCCAGGGA TCTTAGCAAC ATCAATTGTG AAGAGCTTGG TCCATTGCCT

       910        920        930        940        950
          *          *          *          *          *
CCTGGATGGG AGATCCGTAA TACGGCAACA GGCAGAGTTT ATTTCGTTGA

       960        970        980        990       1000
          *          *          *          *          *
CCATAACAAC AGAACAACAC AATTTACAGA TCCTCGGCTG TCTGCTAACT

      1010       1020       1030       1040       1050
          *          *          *          *          *
TGCATTTAGT TTTAAATCGG CAGAACCAAT TGAAAGACCA ACAGCAACAG

      1060       1070       1080       1090       1100
          *          *          *          *          *
CAAGTGGTAT CGTTATGTCC TGATGACACA GAATGCCTGA CAGTCCCAAG

      1110       1120       1130       1140       1150
          *          *          *          *          *
GTACAAGCGA GACCTGGTTC AGAAACTAAA AATTTTGCGG CAAGAACTTT

      1160       1170       1180       1190       1200
          *          *          *          *          *
CCCAACAACA GCCTCAGGCA GGTCATTGCC GCATTGAGGT TTCCAGGGAA

      1210       1220       1230       1240       1250
          *          *          *          *          *
GAGATTTTTG AGGAATCATA TCGACAGGTC ATGAAAATGA GACCAAAAGA

      1260       1270       1280       1290       1300
          *          *          *          *          *
TCTCTGGAAG CGATTAATGA TAAAATTTCG TGGAGAAGAA GGCCTTGACT

      1310       1320       1330       1340       1350
          *          *          *          *          *
ATGGAGGCGT TGCCAGGGAA TGGTTGTATC TCTTGTCACA TGAAATGTTG

      1360       1370       1380       1390       1400
          *          *          *          *          *
AATCCATACT ATGGCCTCTT CCAGTATTCA AGAGATGATA TTTATACATT

      1410       1420       1430       1440       1450
          *          *          *          *          *
```

FIGURE 11

```
GCAGATCAAT CCTGATTCTG CAGTTAATCC GGAACATTTA TCCTATTTCC

      1460        1470        1480        1490        1500
         *           *           *           *           *
ACTTTGTTGG ACGAATAATG GGAATGGCTG TGTTTCATGG ACATTATATT

      1510        1520        1530        1540        1550
         *           *           *           *           *
GATGGTGGTT TCACATTGCC TTTTTATAAG CAATTGCTTG GGAAGTCAAT

      1560        1570        1580        1590        1600
         *           *           *           *           *
TACCTTGGAT GACATGGAGT TAGTAGATCC GGATCTTCAC AACAGTTTAG

      1610        1620        1630        1640        1650
         *           *           *           *           *
TGTGGATACT TGAGAATGAT ATTACAGGTG TTTTGGACCA TACCTTCTGT

      1660        1670        1680        1690        1700
         *           *           *           *           *
GTTGAACATA ATGCATATGG TGAAATTATT CAGCATGAAC TTAAACCAAA

      1710        1720        1730        1740        1750
         *           *           *           *           *
TGGCAAAAGT ATCCCTGTTA ATGAAGAAAA TAAAAAAGAA TATGTCAGGC

      1760        1770        1780        1790        1800
         *           *           *           *           *
TCTATGTGAA CTGGAGATTT TTACGAGGCA TTGAGGCTCA ATTCTTGGCT

      1810        1820        1830        1840        1850
         *           *           *           *           *
CTGCAGAAAG GATTTAATGA AGTAATTCCA CAACATCTGC TGAAGACATT

      1860        1870        1880        1890        1900
         *           *           *           *           *
TGATGAGAAG GAGTTAGAGC TCATTATTTG TGGACTTGGA AAGATAGATG

      1910        1920        1930        1940        1950
         *           *           *           *           *
TTAATGACTG GAAGGTAAAC ACCCGGTTAA AACACTGTAC ACCAGACAGC

      1960        1970        1980        1990        2000
         *           *           *           *           *
AACATTGTCA AATGGTTCTG GAAAGCTGTG GAGTTTTTTG ATGAAGAGCG

      2010        2020        2030        2040        2050
         *           *           *           *           *
ACGAGCAAGA TTGCTTCAGT TTGTGACAGG ATCCTCTCGA GTGCCTCTGC

      2060        2070        2080        2090        2100
         *           *           *           *           *
AGGGCTTCAA AGCATTGCAA GGTGCTGCAG GCCCGAGACT CTTTACCATA

      2110        2120        2130        2140        2150
         *           *           *           *           *
CACCAGATTG ATGCCTGCAC TAACAACCTG CCGAAAGCCC ACACTTGCTT
```

FIGURE 11

```
      2160        2170        2180        2190        2200
       *           *           *           *           *
CAATCGAATA GACATTCCAC CCTATGAAAG CTATGAAAAG CTATATGAAA

      2210        2220        2230        2240
       *           *           *           *
AGCTGCTAAC AGCCATTGAA GAAACATGTG GATTTGCTGT GGAATGA
```

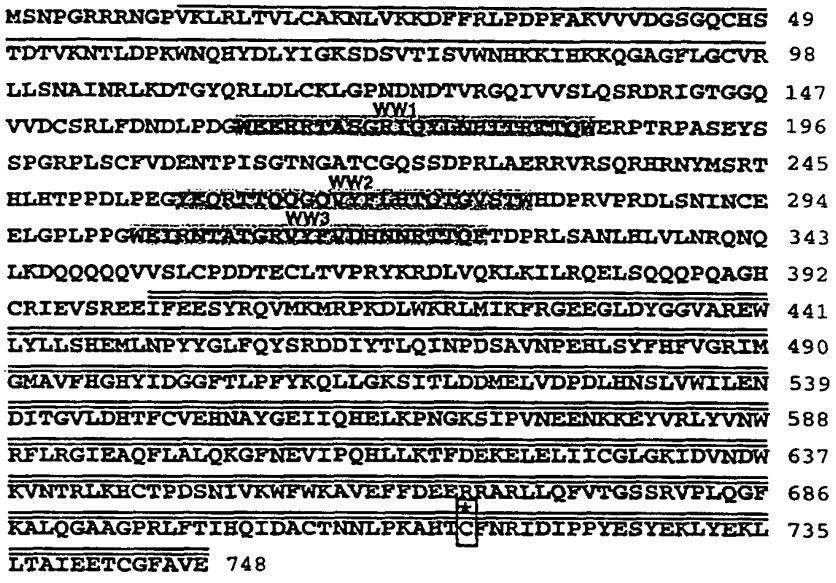


FIGURE 12

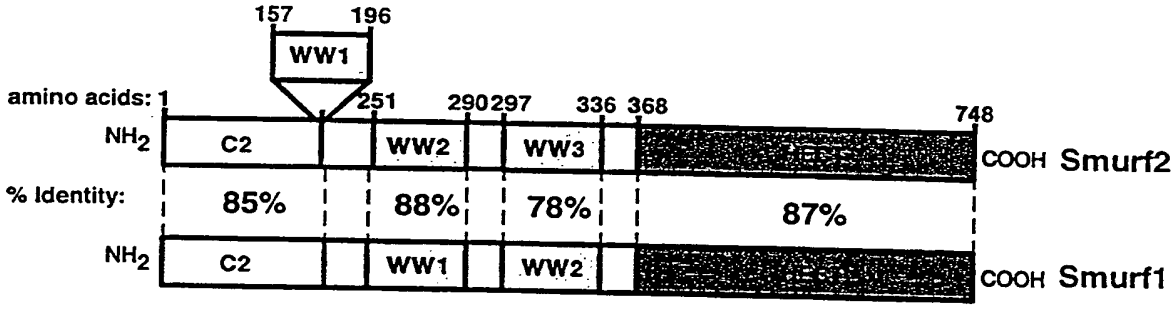
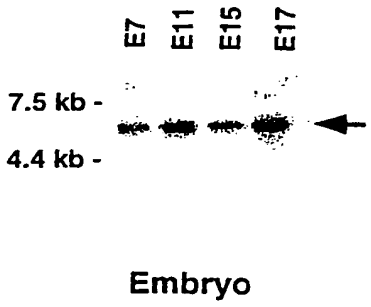
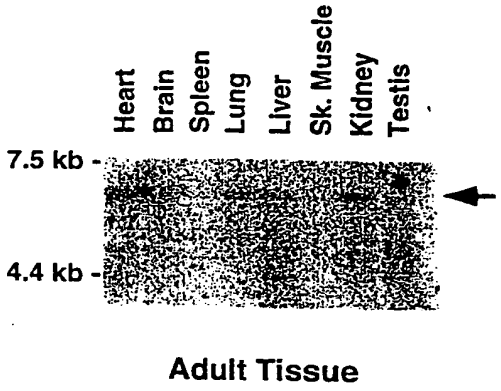

157
196
WW1
amino acids: 1
251
290
297
336
368
748
NH2
C2
WW2
WW3
COOH Smurf2
% Identity:
85%
88%
78%
87%
NH2
C2
WW1
WW2
COOH Smurf1

FIGURE 13


E7
E11
E15
E17
7.5 kb -
4.4 kb -
Embryo

A


Heart
Brain
Spleen
Lung
Liver
Sk. Muscle
Kidney
Testis
7.5 kb -
4.4 kb -
Adult Tissue

B

FIGURE 14

FIGURE 15

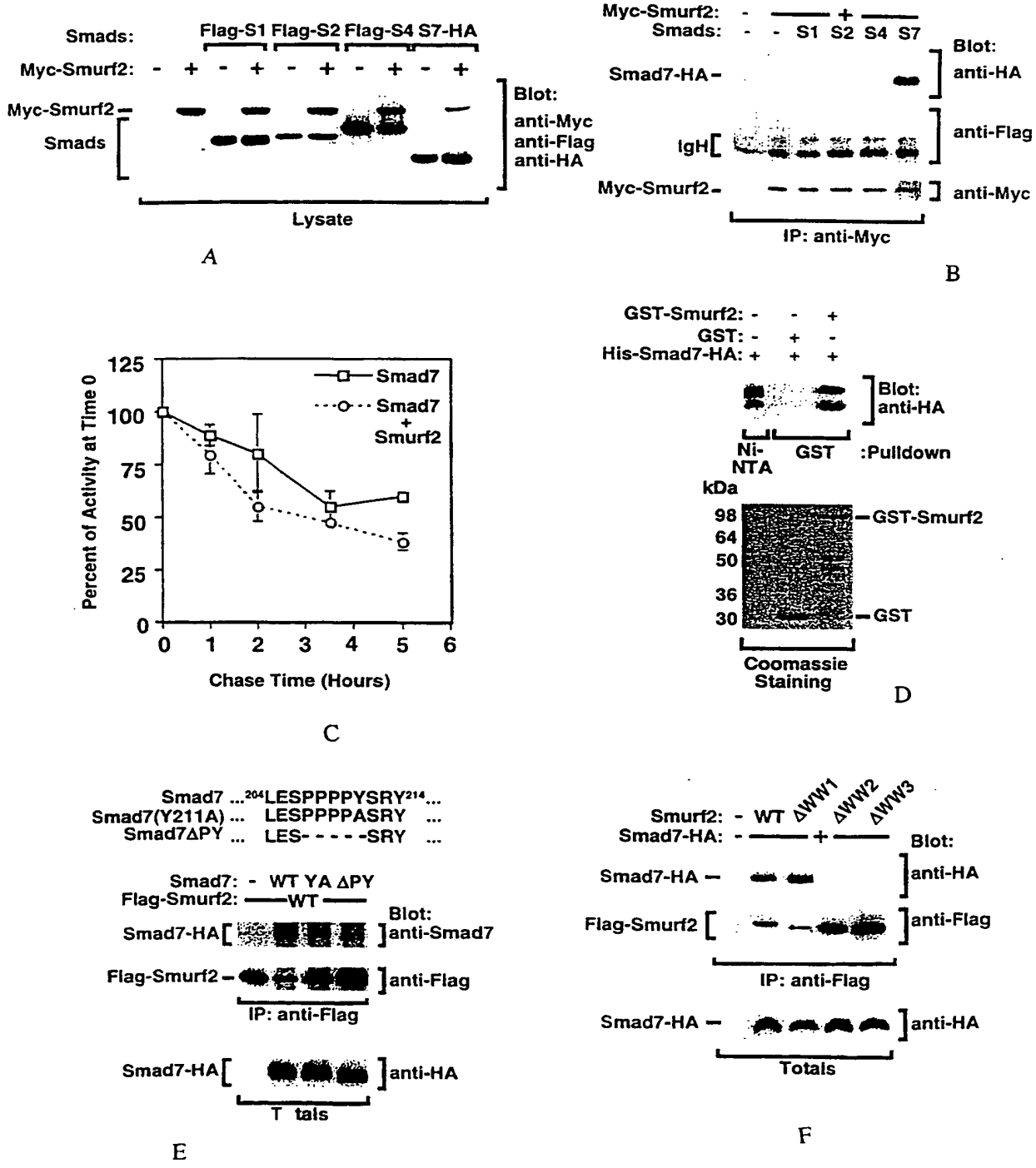

Smads:
Flag-S1 Flag-S2 Flag-S4 S7-HA
Myc-Smurf2: - + - + - + - + - +
Myc-Smurf2
Smads
Blot:
anti-Myc
anti-Flag
anti-HA
Lysate
A
Myc-Smurf2: - +
Smads: - - S1 S2 S4 S7
Blot:
Smad7-HA
anti-HA
IgH
anti-Flag
Myc-Smurf2
anti-Myc
IP: anti-Myc
B
Percent of Activity at Time 0
125
100
75
50
25
0
0 1 2 3 4 5 6
Chase Time (Hours)
Smad7
Smad7 + Smurf2
C
GST-Smurf2: - - +
GST: - + -
His-Smad7-HA: + + +
Blot:
anti-HA
Ni-NTA
GST
:Pulldown
kDa
98
64
50
36
30
GST-Smurf2
GST
Coomassie Staining
D
Smad7 ...204LESPPPPYSRY214...
Smad7(Y211A) ... LESPPPPASRY ...
Smad7ΔPY ... LES- - - - -SRY ...
Smad7: - WT YA ΔPY
Flag-Smurf2: —WT—
Blot:
Smad7-HA
anti-Smad7
Flag-Smurf2
anti-Flag
IP: anti-Flag
Smad7-HA
anti-HA
T tals
E
Smurf2: - WT ΔWW1 ΔWW2 ΔWW3
Smad7-HA: - + Blot:
Smad7-HA
anti-HA
Flag-Smurf2
anti-Flag
IP: anti-Flag
Smad7-HA
anti-HA
Totals
F

FIGURE 16

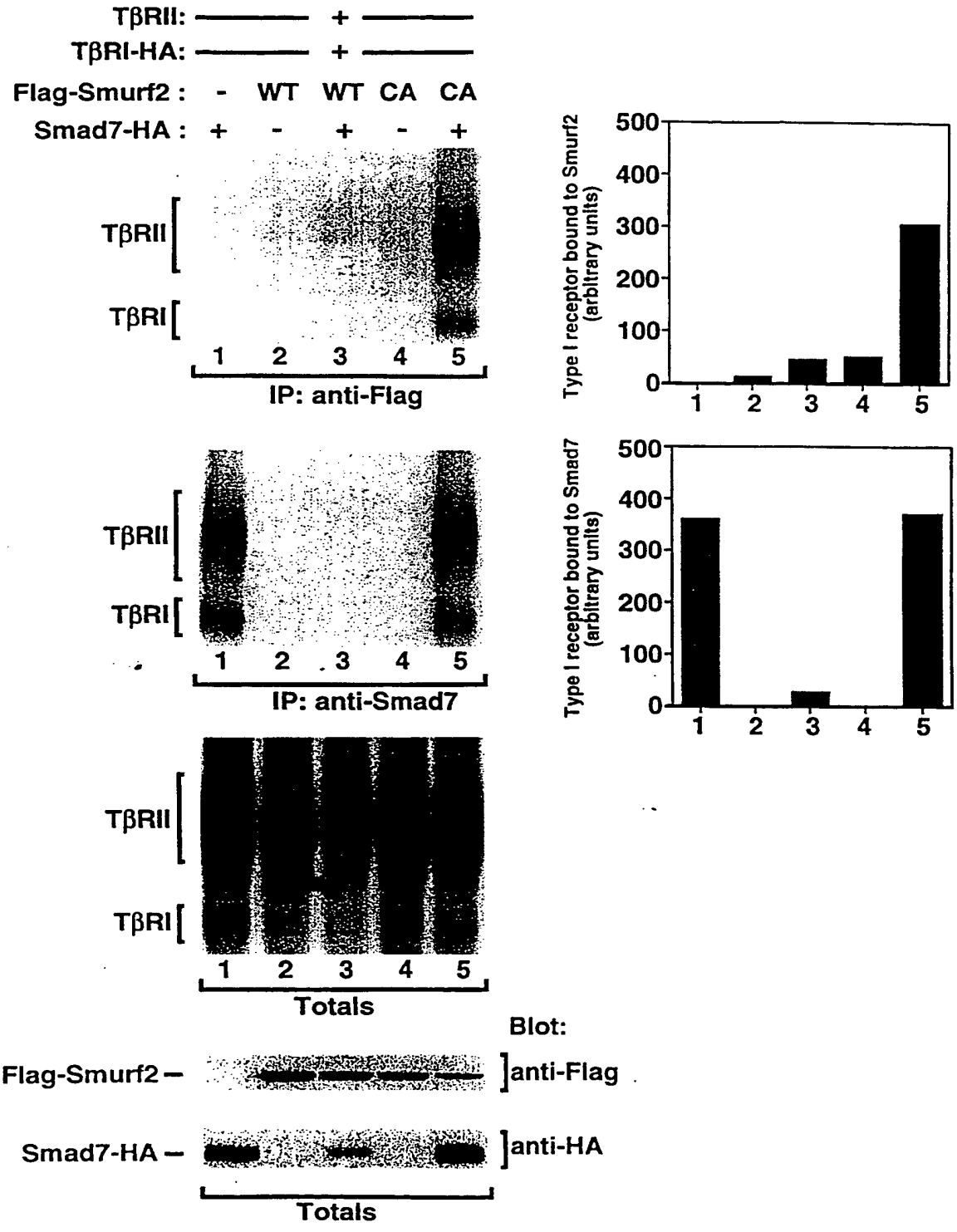

TβRII: — + —
TβRI-HA: — + —
Flag-Smurf2 : - WT WT CA CA
Smad7-HA : + - + - +
TβRII
TβRI
1 2 3 4 5
IP: anti-Flag
Type I receptor bound to Smurf2 (arbitrary units)
500
400
300
200
100
0
1 2 3 4 5
TβRII
TβRI
1 2 3 4 5
IP: anti-Smad7
Type I receptor bound to Smad7 (arbitrary units)
500
400
300
200
100
0
1 2 3 4 5
TβRII
TβRI
1 2 3 4 5
Totals
Blot:
Flag-Smurf2
anti-Flag
Smad7-HA
anti-HA
Totals

FIGURE 17

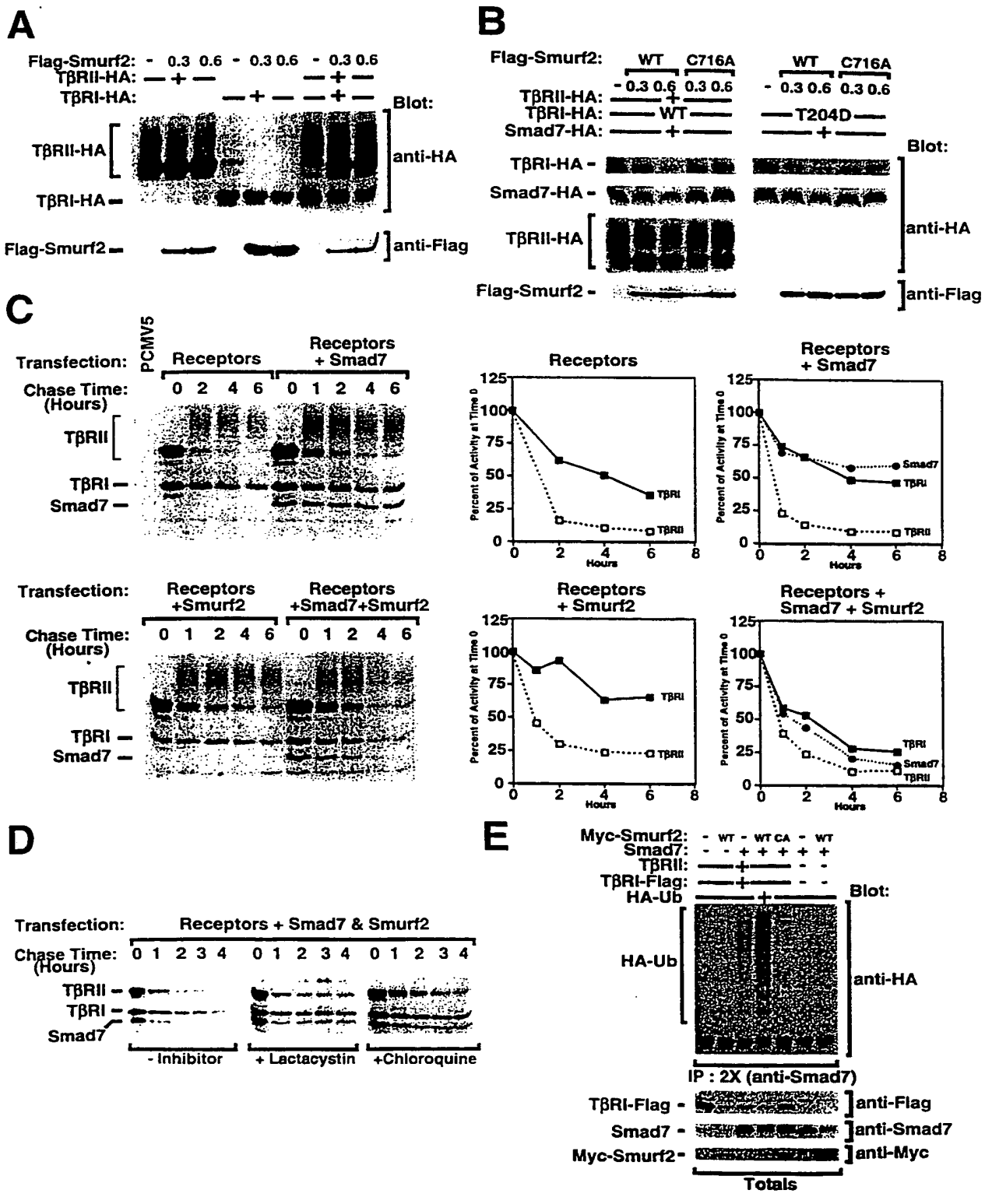
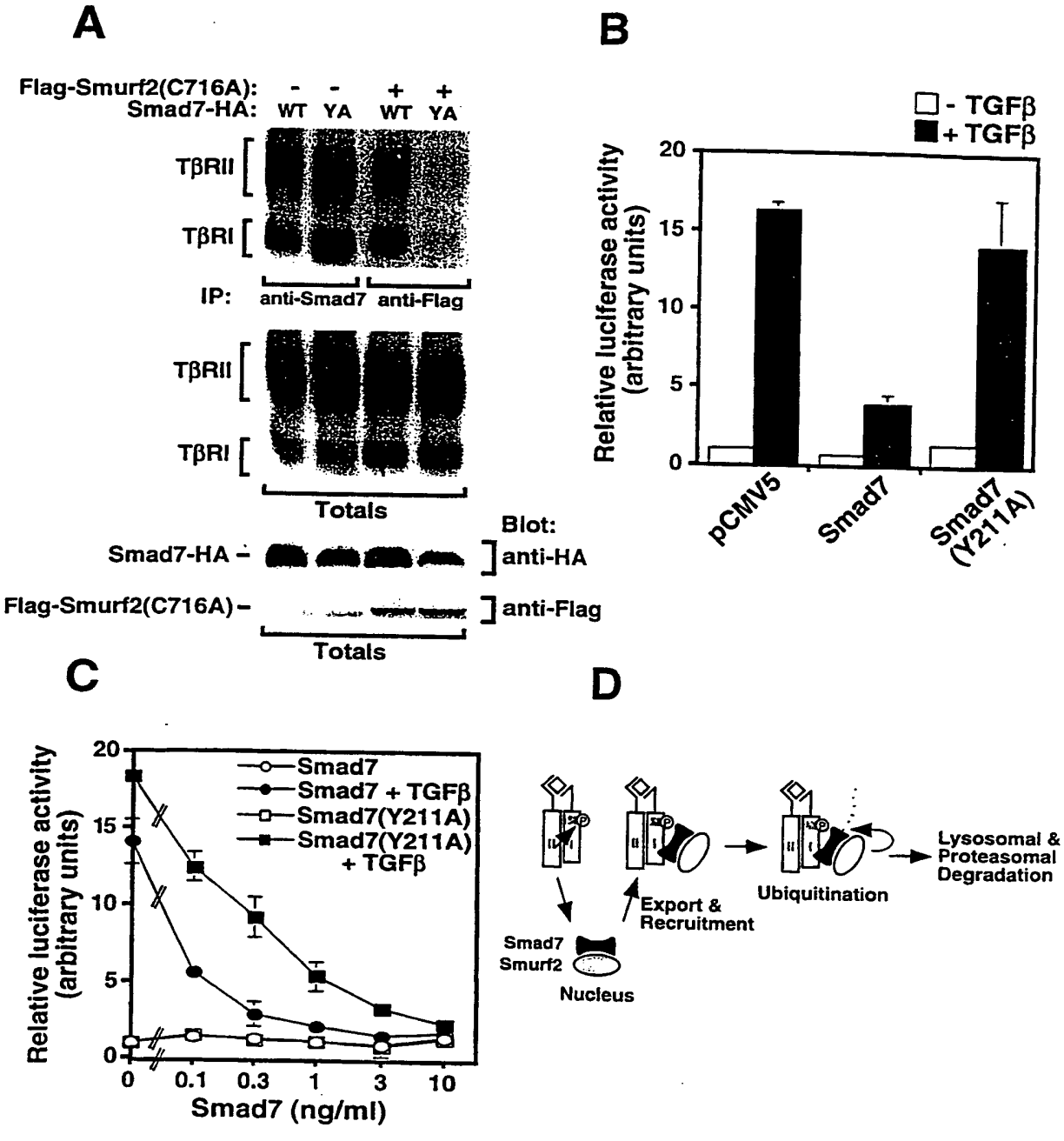

A
Flag-Smurf2: - 0.3 0.6 - 0.3 0.6 - 0.3 0.6
TβRII-HA:
TβRI-HA:
Blot:
TβRII-HA
TβRI-HA
anti-HA
Flag-Smurf2
anti-Flag
B
Flag-Smurf2: WT C716A WT C716A
- 0.3 0.6 0.3 0.6 - 0.3 0.6 0.3 0.6
TβRII-HA:
TβRI-HA: WT T204D
Smad7-HA:
Blot:
TβRI-HA
Smad7-HA
TβRII-HA
anti-HA
Flag-Smurf2
anti-Flag
C
Transfection: PCMV5 Receptors Receptors + Smad7
Chase Time: (Hours) 0 2 4 6 0 1 2 4 6
TβRII
TβRI
Smad7
Transfection: Receptors +Smurf2 Receptors +Smad7+Smurf2
Chase Time: (Hours) 0 1 2 4 6 0 1 2 4 6
TβRII
TβRI
Smad7
Receptors
Receptors + Smad7
Receptors + Smurf2
Receptors + Smad7 + Smurf2
Percent of Activity at Time 0
Hours
TβRI
TβRII
Smad7
D
Transfection: Receptors + Smad7 & Smurf2
Chase Time: (Hours) 0 1 2 3 4 0 1 2 3 4 0 1 2 3 4
TβRII
TβRI
Smad7
- Inhibitor
+ Lactacystin
+Chloroquine
E
Myc-Smurf2: - WT - WT CA - WT
Smad7: - - + + + + +
TβRII:
TβRI-Flag:
HA-Ub
Blot:
HA-Ub
anti-HA
IP : 2X (anti-Smad7)
TβRI-Flag
anti-Flag
Smad7
anti-Smad7
Myc-Smurf2
anti-Myc
Totals

FIGURE 18


A
Flag-Smurf2(C716A): - - + +
Smad7-HA: WT YA WT YA
TβRII
TβRI
IP: anti-Smad7 anti-Flag
TβRII
TβRI
Totals
Blot:
Smad7-HA
anti-HA
Flag-Smurf2(C716A)
anti-Flag
Totals
B
- TGFβ
+ TGFβ
Relative luciferase activity (arbitrary units)
20
15
10
5
0
pCMV5
Smad7
Smad7 (Y211A)
C
Relative luciferase activity (arbitrary units)
Smad7
Smad7 + TGFβ
Smad7(Y211A)
Smad7(Y211A) + TGFβ
0 0.1 0.3 1 3 10
Smad7 (ng/ml)
D
Lysosomal & Proteasomal Degradation
Ubiquitination
Export & Recruitment
Smad7
Smurf2
Nucleus

0974/2E916 WO

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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International Bureau

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(25) Filing Language: English

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(30) Priority Data:
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(71) Applicants *(for all designated States except US)*: **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK** [US/US]; Stony Brook, NY 11794-3366 (US). **HSC RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP** [CA/CA]; 555 University Avenue, Toronto, Ontario M5G 1X8 (CA).

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(74) Agents: **SEMIONOW, Raina** et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*

(88) Date of publication of the international search report:
3 May 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/77168 A3

(54) Title: ANTAGONISTS OF BMP AND TGFβ SIGNALLING PATHWAYS

(57) Abstract: This invention provides unique members of the Hect family of ubiquitin ligases that specifically target BMP and TGFβ/activin pathway-specific Smads. The novel ligases have been named Smurf1 and Smurf2. They directly interact with Smads1 and 5 and Smad7, respectively, and regulate the ubiquitination, turnover and activity of Smads and other proteins of these pathways. Smurf1 interferes with biological responses to BMP, but not activin signalling. In amphibian embryos Smurf1 inhibits endogenous BMP signals, resulting in altered pattern formation and cell fate specification in the mesoderm and ectoderm. The present invention provides a unique regulatory link between the ubiquitination pathway and the control of cell fate determination by the TGFβ superfamily during embryonic development. Thus, Smurf1 is a negative regulator of Smad1 signal transduction, by targeting Smad1, Smurf1 blocks BMP signalling. In mammalian cells, Smurf2 suppresses TGFβ signalling, and in Xenopus, blocks formation of dorsal mesoderm and causes anterior truncation of the embryos. Smurf2 forms a stable complex with Smad7, which induces degradation and downregulation of TGFβ/activin signalling.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16250

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C07H 21/04; C12N 15/00; C07K 14/00.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 329, 328, 300, 326, 327; 435/29, 320.1, 325, 375; 536/23.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: BIOSIS, MEDLINE, DGENE, BIOTECHDS, BIOTECHABS, HCAPLUS, USPAT, WPIDS, SCISEARCH, EMBASE.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 6,087,122 A (HUSTAD et al) 11 July 2000 (11/7/00). See entire document.	1-4, 7, 8, 11-14, 18, 19 and 23-26, 36
Y,E	US 6,103,869 A (SOUCHELNYTOKYI ET AL) 15 August 2000 (15/8/00). See entire document.	1-4, 7, 8, 11-14, 18, 19 and 23-26, 36
Y,E	WRANA et al. The Smad Pathway. Cytokine And Growth Factor Reviews. 2000, Vol. 11, pages 5-13, see entire document.	1-4, 7, 8, 11-14, 18, 19, 23-26, 36

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 NOVEMBER 2000	Date of mailing of the international search report 15 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HOPE ROBINSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16250

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-26 and 36

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PATENT ABSTRACTS OF JAPAN

(11)Publication number : 07-064134
(43)Date of publication of application : 10.03.1995

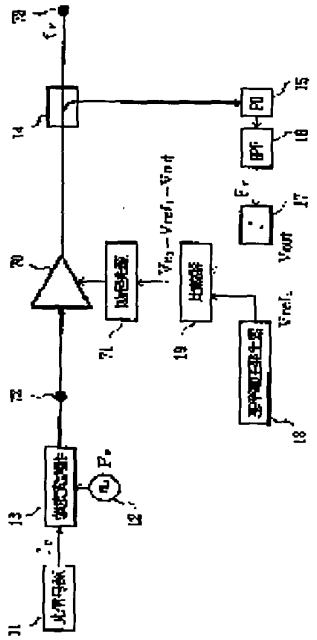
(51)Int.Cl. G02F 1/35
H04B 10/14
H04B 10/06
H04B 10/04

(21)Application number : 05-207989
(22)Date of filing : 23.08.1993

(71)Applicant : NIPPON TELEGR & TELEPH CORP <NTT>
(72)Inventor : ODA KAZUHIRO
TOBA HIROSHI

(54) OUTPUT CONTROL CIRCUIT FOR OPTICAL AMPLIFIER

(57)Abstract:
PURPOSE: To control an output level or optical amplification gain so as to stabilize this level or gain without receiving the influence of the naturally released light noises included in the output light of an optical amplifier by providing the above control circuit with an output level control means for setting the optical amplification gain of the optical amplifier according to modulation signal intensity.
CONSTITUTION: An intensity modulator 13 modulates the intensity of the light signal outputted from a light source 11 by the modulation signal outputted from a low frequency oscillator 13. This intensity modulated light is inputted to the optical amplifier 70. A part of the output light of the optical amplifier 70 is branched by an optical branching element 14 and is received in a photodetector 15. This output control circuit is so constituted that part of the output light of the optical amplifier 70 is detected by the photocletector 15 and that the modulation signal of the frequency Fe filtered by a band-pass filter 16 is monitored. Then, the output level of the optical amplifier 70 is selectively controlled for the light frequency band corresponding to the light frequency fe of the light signal source without substantially receiving the influence of the naturally released light noises widely distributed on the light frequency band of the optical amplifier 70.



LEGAL STATUS

[Date of request for examination]

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

(19)日本国特許庁（ＪＰ）　(12)公開特許公報（Ａ）　(11)特許出願公開番号

特開平7－64134

(43)公開日　平成７年(1995)３月10日

(51)Int.Cl.[6]		識別記号	庁内整理番号	Ｆ Ｉ	技術表示箇所
Ｇ０２Ｆ	1/35	５０１	9316－2K		
Ｈ０４Ｂ	10/14				
	10/06				
	10/04				
			9372－5K	Ｈ０４Ｂ　9/ 00	Ｓ

審査請求　未請求　請求項の数４　ＯＬ　（全 13 頁）

(21)出願番号　特願平5－207989

(22)出願日　平成５年(1993)８月23日

(71)出願人　000004226
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(72)発明者　鳥羽　弘
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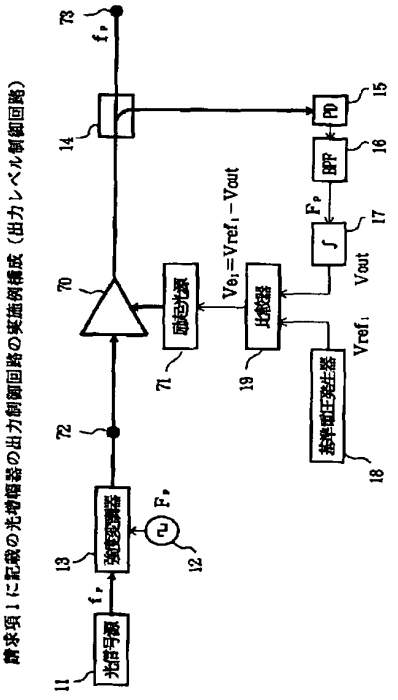
(74)代理人　弁理士　古谷　史旺

(54)【発明の名称】　光増幅器の出力制御回路

(57)【要約】

【目的】　光増幅器の出力制御回路に関し、光増幅器の出力光に含まれる自然放出光雑音の影響を受けず、また光周波数多重化された光信号が入力される場合でも、入力光信号の多重数の変動および光増幅利得特性の光周波数依存性の影響を受けずに、出力レベルあるいは光増幅利得を安定制御することを目的とする。

【構成】　入力光を光増幅する光増幅器の出力レベルを所定値に制御する光増幅器の出力制御回路において、所定の光周波数帯の光を所定の周波数の変調信号で強度変調し、その強度変調光を光増幅器に入力させる強度変調光入力手段と、光増幅器の出力光の一部を受光し、受光強度に比例する信号を出力する受光手段と、受光手段の出力信号から所定の周波数の変調信号を検出し、その信号強度を測定する変調信号強度測定手段と、変調信号強度に応じて、光増幅器の光増幅利得を設定する光増幅利得制御手段とを備える。



【特許請求の範囲】

【請求項１】　入力光を光増幅する光増幅器の出力レベルを所定値に制御する光増幅器の出力制御回路において、

所定の光周波数帯の光を所定の周波数の変調信号で強度変調し、その強度変調光を前記光増幅器に入力させる強度変調光入力手段と、

前記光増幅器の出力光の一部を受光し、受光強度に比例する信号を出力する受光手段と、

前記受光手段の出力信号から前記所定の周波数の変調信号を検出し、その信号強度を測定する変調信号強度測定手段と、

前記変調信号強度に応じて、前記光増幅器の光増幅利得を設定する出力レベル制御手段とを備えたことを特徴とする光増幅器の出力制御回路。

【請求項２】　入力光を光増幅する光増幅器の光増幅利得を所定値に制御する光増幅器の出力制御回路において、

所定の光周波数帯の光を所定の周波数の変調信号で強度変調し、その強度変調光を前記光増幅器に入力させる強度変調光入力手段と、

前記光増幅器の入力光の一部を受光し、受光強度に比例する信号を出力する入力段受光手段と、

前記入力段受光手段の出力信号から前記所定の周波数の変調信号を検出し、その信号強度を測定する入力段変調信号強度測定手段と、

前記光増幅器の出力光の一部を受光し、受光強度に比例する信号を出力する出力段受光手段と、

前記出力段受光手段の出力信号から前記所定の周波数の変調信号を検出し、その信号強度を測定する出力段変調信号強度測定手段と、

前記各変調信号強度から、前記所定の光周波数帯における光増幅利得を算出する光増幅利得算出手段と、

前記所定の光周波数帯における光増幅利得に応じて、前記光増幅器の光増幅利得を設定する光増幅利得制御手段とを備えたことを特徴とする光増幅器の出力制御回路。

【請求項３】　光周波数多重された入力光を光増幅する光増幅器の出力レベルを所定値に制御する光増幅器の出力制御回路において、

複数の光周波数帯の光をそれぞれ対応する所定の周波数の変調信号で強度変調し、各強度変調光を多重化して前記光増幅器に入力させる強度変調光入力手段と、

前記光増幅器の出力光を入力し、前記各光周波数帯ごとに出力レベルを調整する光利得等化回路と、

前記光利得等化回路の出力光の一部を受光し、受光強度に比例する信号を出力する受光手段と、

前記受光手段の出力信号から前記各周波数の変調信号を検出し、それぞれの信号強度を測定する変調信号強度測定手段と、

前記各変調信号強度に応じて、前記光増幅器の光増幅利得および前記光利得等化回路における各光周波数帯の出力レベルを設定する出力レベル制御手段とを備えたことを特徴とする光増幅器の出力制御回路。

【請求項４】　光周波数多重された入力光を光増幅する光増幅器の光増幅利得を所定値に制御する光増幅器の出力制御回路において、

複数の光周波数帯の光をそれぞれ対応する所定の周波数の変調信号で強度変調し、各強度変調光を多重化して前記光増幅器に入力させる強度変調光入力手段と、

前記光増幅器の入力光の一部を受光し、受光強度に比例する信号を出力する入力段受光手段と、

前記入力段受光手段の出力信号から前記各周波数の変調信号を検出し、それぞれの信号強度を測定する入力段変調信号強度測定手段と、

前記光増幅器の出力光を入力し、前記各光周波数帯ごとに出力レベルを調整する光利得等化回路と、

前記光利得等化回路の出力光の一部を受光し、受光強度に比例する信号を出力する出力段受光手段と、

前記出力段受光手段の出力信号から前記各周波数の変調信号を検出し、それぞれの信号強度を測定する出力段変調信号強度検出手段と、

前記各変調信号強度から、前記各光周波数帯における光増幅利得を算出する光増幅利得算出手段と、

前記各光周波数帯における光増幅利得に応じて、前記光増幅器の光増幅利得および前記光利得等化回路における各光周波数帯の出力レベルを設定する光増幅利得制御手段とを備えたことを特徴とする光増幅器の出力制御回路。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は、光信号を増幅する光増幅器の出力制御回路に関する。なお、光増幅器の出力制御には、出力レベルを安定化する出力レベル制御と、光増幅利得を一定にする利得制御とを含む。

【０００２】

【従来の技術】図７は、従来の光増幅器の出力レベル制御回路の構成例を示す。図において、光増幅器７０は、励起光源７１から出力される励起光の作用により、光信号入力端子７２に入力された光信号を増幅して光信号出力端子７３に送出する。この光増幅器７０の出力レベルを安定化する出力レベル制御回路は、以下のように構成される。

【０００３】光増幅器７０の出力光の一部は、光分岐素子７４で分岐して受光素子（ＰＤ）７５に受光される。受光素子７５は受光強度に比例する信号を出力し、増幅器７６を介して比較器７７に与える。比較器７７は、その信号電圧V_1と基準電圧発生器７８から与えられる基準電圧V_0とを比較し、その差分を示す誤差電圧Ve（$=V_0-V_1$）を出力する。この誤差電圧Veは、光増幅器７０の出力光強度が所定値よりも大きいときには負

となり、小さいときには正となり、両者が等しいときには０となる。比較器７７で得られた誤差電圧Ｖｅは、低域通過フィルタ（ＬＰＦ）７９で不要な高周波成分が除去されて光増幅器７０の励起光源７１に帰還する。励起光源７１は、バイアス電流値に応じて励起光強度が増減するので、バイアス電流値を誤差電圧Ｖｅに応じて調整することにより、光増幅器７０の出力レベルを安定化させることができる。

【０００４】図８は、励起光源７１から出力される励起光強度と光増幅器７０の出力光強度の関係を示す。励起光強度の増大に応じて、光増幅器７０の出力光強度（あるいは光増幅利得）が増大することがわかる。すなわち、励起光源７１を制御して励起光強度を増減すれば、光増幅器７０の出力光強度（あるいは光増幅利得）を調整できることがわかる。

【０００５】このように、光増幅器の出力レベル制御回路は、光増幅器７０の出力光の一部を分岐し、受光素子７５によりその光強度に比例した信号を検出し、所定のレベルとの差分を光増幅器７０の励起光源７１に帰還することにより、光増幅器７０の出力レベルを基準電圧V_0に応じたレベルに安定化する構成になっている。

【０００６】

【発明が解決しようとする課題】ところで、従来の出力レベル制御回路は、光増幅器の総出力光強度を一定に保持するように機能している。一方、光増幅器の出力光の中には、入力光信号が増幅されたものと、自然放出光による雑音成分が混在している。したがって、雑音込みで光増幅器の出力レベルが制御される構成になっていた。これは、増幅された光信号成分が自然放出光成分に比べて十分に大きいときには問題にならないが、そうでないときには雑音電力分だけずれた出力レベルに安定化されることになる。

【０００７】また、従来の光増幅器の出力レベル制御回路では、光周波数多重化された光信号を増幅する場合には次の問題点が生ずる。

① 入力光信号の多重数が変動すると光増幅器の飽和レベルが変動するので、見かけ上の総出力光強度を一定に制御すると、各チャネルの出力光強度がそれにつれて変動することになる。

【０００８】② 実際の光増幅器の利得特性は、入力光信号の光周波数に依存して変化するので、見かけ上の総出力光強度を一定に制御しても、各チャネルの出力レベルあるいは光増幅利得が一定になるとは限らない。

【０００９】本発明は、光増幅器の出力光に含まれる自然放出光雑音の影響を受けない光増幅器の出力制御回路、また光周波数多重化された光信号が入力される場合でも、入力光信号の多重数の変動および光増幅利得特性の光周波数依存性の影響を受けない光増幅器の出力制御回路を提供することを目的とする。

【００１０】

【課題を解決するための手段】請求項１に記載の発明は、入力光を光増幅する光増幅器の出力レベルを所定値に制御する光増幅器の出力制御回路において、所定の光周波数帯の光を所定の周波数の変調信号で強度変調し、その強度変調光を光増幅器に入力させる強度変調光入力手段と、光増幅器の出力光の一部を受光し、受光強度に比例する信号を出力する受光手段と、受光手段の出力信号から所定の周波数の変調信号を検出し、その信号強度を測定する変調信号強度測定手段と、変調信号強度に応じて、光増幅器の光増幅利得を設定する出力レベル制御手段とを備える。

【００１１】請求項２に記載の発明は、請求項１に記載の発明の構成に加えて、光増幅器の入力光の一部を受光し、受光強度に比例する信号を出力する入力段受光手段と、入力段受光手段の出力信号から所定の周波数の変調信号を検出し、その信号強度を測定する入力段変調信号強度測定手段と、入出力段で測定される各変調信号強度から、所定の光周波数帯における光増幅利得を算出する光増幅利得算出手段と、所定の光周波数帯における光増幅利得に応じて、光増幅器の光増幅利得を設定する光増幅利得制御手段とを備える。

【００１２】請求項３に記載の発明は、請求項１に記載の発明において、複数の光周波数帯の強度変調光を用い、各変調信号強度に応じて光増幅器の出力レベルを制御することを特徴とする。

【００１３】請求項４に記載の発明は、請求項２に記載の発明において、複数の光周波数帯の強度変調光を用い、入出力段で測定される各変調信号強度に応じて光増幅器の光増幅利得を制御することを特徴とする。

【００１４】

【作用】請求項１に記載の光増幅器の出力制御回路では、制御対象となる光周波数帯の光を所定の周波数の変調信号で強度変調し、その強度変調光を光増幅器に入力する。さらに、光増幅器の出力光の一部を受光し、所定の周波数の変調信号を検出してその信号強度を測定する。この変調信号強度に応じて光増幅器の光増幅利得を設定することにより、希望する光周波数帯の入力光について、光増幅器の出力レベルを安定化することができる。

【００１５】請求項２に記載の光増幅器の出力制御回路では、同様に光増幅器の入力光から変調信号強度を検出し、出力光から得られる変調信号強度との対比から所定の光周波数帯における光増幅利得を算出する。この光増幅利得に応じて光増幅器の光増幅利得を設定することにより、希望する光周波数帯の入力光について、光増幅器の光増幅利得を一定に保持することができる。

【００１６】このような強度変調光をモニタして変調信号強度を測定する構成では、光増幅器の光周波数帯域上に広く分布する自然放出光雑音の影響をほとんど受けずにすむ。すなわち、光増幅器の出力に含まれる自然放出

光雑音の影響を受けない光増幅器の出力制御回路を実現することができる。

【００１７】請求項３および請求項４に記載の光増幅器の出力制御回路は、光周波数多重された入力光を光増幅する光増幅器に適用される。すなわち、複数の光周波数帯の光をそれぞれ所定の周波数の変調信号で強度変調し、各強度変調光を多重化して光増幅器に入力する。以下同様に、各光周波数帯（チャネル）の強度変調光をモニタし、それぞれの変調信号強度に応じて各チャネルごとに光利得等化処理を行う。これにより、各チャネルの光信号について光増幅器の出力レベルあるいは光増幅利得を安定化することができる。

【００１８】したがって、その多重数が変動する場合、あるいは光増幅器の光増幅利得特性が光周波数依存性を有する場合でも、各チャネルの光信号について光増幅器の出力レベルあるいは光増幅利得を安定化することができる。すなわち、入力光信号の多重数の変動および光増幅利得特性の光周波数依存性の影響を受けない光増幅器の出力制御回路を実現することができる。

【００１９】

【実施例】図１は、請求項１に記載の光増幅器の出力制御回路の実施例構成を示す。なお、本実施例は、光増幅器の出力レベルを安定化する出力レベル制御回路としての構成を示す。

【００２０】図において、光増幅器７０は、励起光源７１から出力される励起光の作用により、光信号入力端子７２から入力された光信号を増幅して光信号出力端子７３に送出する。

【００２１】この光増幅器７０の出力レベルを安定化する本実施例の出力制御回路（出力レベル制御回路）は、光信号源１１から出力される光周波数 f_p の光信号を強度変調する周波数 F_p の変調信号を発生する低周波発振器１２、この変調信号で駆動される強度変調器１３、光分岐素子１４、受光素子（ＰＤ）１５、変調信号の周波数 F_p を中心周波数とする帯域通過フィルタ（ＢＰＦ）１６、包絡線検波器１７、基準電圧 $Vref_1$ を発生する基準電圧発生器１８、比較器１９により構成される。

【００２２】なお、低周波発振器１２および強度変調器１３は、強度変調光入力手段に対応する。光分岐素子１４および受光素子１５は、受光手段に対応する。帯域通過フィルタ１６および包絡線検波器１７は、変調信号強度測定手段に対応する。基準電圧発生器１８，比較器１９および励起光源７１は、出力レベル制御手段に対応する。

【００２３】強度変調器１３は、光信号源１１から出力された光信号を低周波発振器１２から出力された変調信号で強度変調し、その強度変調光は光増幅器７０に入力される。光増幅器７０の出力光の一部は、光分岐素子１４で分岐して受光素子１５に受光される。受光素子１５は受光強度に比例する信号を出力し、その出力信号は帯域通過フィルタ１６を介して包絡線検波器１７に入力される。包絡線検波器１７は、光増幅器７０の出力光強度に比例した変調信号電圧Ｖout を検出して比較器１９に与える。比較器１９は、変調信号電圧Ｖout と基準電圧発生器１８から与えられる基準電圧 $Vref_1$ とを比較し、その差分を示す誤差電圧 Ve_1 （＝$Vref_1$－Ｖout ）を出力する。この誤差電圧 Ve_1 は、光増幅器７０の出力光強度が所定値よりも大きいときには負となり、小さいときには正となり、両者が等しいときには０となる。比較器１９で得られた誤差電圧 Ve_1 は光増幅器７０の励起光源７１に帰還され、そのバイアス電流値を誤差電圧 Ve_1 に応じて増減することにより、光増幅器７０の出力レベルを安定化させる。

【００２４】このように本実施例は、光増幅器７０の出力光の一部を受光素子１５で受光し、帯域通過フィルタ１６で濾波される周波数 F_p の変調信号をモニタする構成になっている。したがって、光増幅器７０の光周波数帯域上に広く分布する自然放出光雑音の影響をほとんど受けずに、光信号源１１の光周波数 f_p に対応する光周波数帯域について、光増幅器７０の出力レベルを選択的に制御することができる。

【００２５】なお、本実施例は、伝送信号で変調された光信号を所定の周波数の変調信号で強度変調する構成をとっているが、伝送信号と変調信号を重畳して変調する構成としても、同様に光増幅器７０の出力レベルを選択的に制御することができる。また、以下に示す実施例においても同様である。

【００２６】図２は、請求項２に記載の光増幅器の出力制御回路の実施例構成を示す。なお、本実施例は、光増幅器の光増幅利得を一定にする利得制御回路としての構成を示す。

【００２７】図において、光増幅器７０は、励起光源７１から出力される励起光の作用により、光信号入力端子７２から入力された光信号を増幅して光信号出力端子７３に送出する。

【００２８】この光増幅器７０の光増幅利得を一定にする本実施例の出力制御回路（利得制御回路）は、図１に示す出力制御回路（出力レベル制御回路）の構成に加えて、光増幅器７０の入力段に設けられる同様の光分岐素子２１、受光素子２２、帯域通過フィルタ（ＢＰＦ）２３、包絡線検波器２４、除算器２５を含む。

【００２９】なお、低周波発振器１２および強度変調器１３は、強度変調光入力手段に対応する。光分岐素子２１および受光素子２２は、入力段受光素子に対応する。帯域通過フィルタ２３および包絡線検波器２４は、入力段変調信号強度測定手段に対応する。光分岐素子１４および受光素子１５は、出力段受光手段に対応する。帯域通過フィルタ１６および包絡線検波器１７は、出力段変調信号強度測定手段に対応する。除算器２５は、光増幅利得算出手段に相当する。基準電圧発生器１８，比較器

１９および励起光源７１は、光増幅利得制御手段に対応する。

【００３０】強度変調器１３は、光信号源１１から出力された光信号を低周波発振器１２から出力された変調信号で強度変調し、その強度変調光は光増幅器７０に入力される。光増幅器７０の入力光の一部は、光分岐素子２１で分岐して受光素子２２に受光される。受光素子２２は受光強度に比例する信号を出力し、その信号は帯域通過フィルタ２３を介して包絡線検波器２４に入力される。包絡線検波器２４は、光増幅器７０の入力光強度に比例した変調信号電圧Ｖinを出力する。

【００３１】一方、光増幅器７０の出力光の一部は、光分岐素子１４で分岐して受光素子１５に受光される。受光素子１５は受光強度に比例する信号を出力し、その信号は帯域通過フィルタ１６を介して包絡線検波器１７に入力される。包絡線検波器１７は、光増幅器７０の出力光強度に比例した変調信号電圧Ｖout を出力する。除算器２５は、光増幅器７０の出力光強度に比例した変調信号電圧Ｖout を入力光強度に比例した変調信号電圧Ｖinで割ることにより、光増幅器７０の光増幅利得に対応する利得電圧Ｖg(＝Ｖout／Ｖin)を得る。比較器１９は、利得電圧Ｖgと基準電圧発生器１８から与えられる基準電圧$Vref_2$とを比較し、その差分を示す誤差電圧Ve_2(＝$Vref_2$－Ｖg) を出力する。この誤差電圧Ve_2は、光増幅器７０の光増幅利得が所定値よりも大きいときには負となり、小さいときには正となり、両者が等しいときには０となる。比較器１９で得られた誤差電圧Ve_2は光増幅器７０の励起光源７１に帰還され、そのバイアス電流値を誤差電圧Ve_2に応じて増減することにより、光増幅器７０の光増幅利得を一定に保持することができる。

【００３２】このように本実施例は、光増幅器７０の入力光および出力光を受光素子２２，１５で検波し、それぞれ帯域通過フィルタ２３，１６で濾波される周波数F_pの変調信号をモニタする構成になっている。したがって、光増幅器７０の光周波数帯域上に広く分布する自然放出光雑音の影響をほとんど受けずに、光信号源１１の光周波数f_pに対応する光周波数帯域について、光増幅器７０の光増幅利得を選択的に制御することができる。

【００３３】ここで、光増幅器７０における強度変調光の増幅例を図３に示す。横軸は光周波数であり、縦軸は光強度である。光増幅器７０の入力光スペクトルおよび出力光スペクトルのピークは光周波数f_pでそれぞれＰin，Ｐout となる。一方、強度変調光は、周波数F_pの変調信号で光強度変調されており、その光電力の変動の振幅は、光増幅器７０の前後においてＡin，Ａout となる。したがって、光増幅器７０の光増幅利得Ｇは、

Ｇ＝Ｐout／Ｐin＝Ａout／Ａin　　…(1)

となる。このような関係から、周波数F_pの変調信号強度を測定することにより光増幅器７０の出力レベルや光増幅利得を制御可能であることがわかる。なお、光増幅器７０の出力光には増幅された自然放出光雑音（ＡＳＥ）も混入するが、光周波数f_pの近傍の帯域内に存在するＡＳＥ電力は比較的小さく、その影響はほとんどない。

【００３４】また、受光素子２２，１５の出力信号の周波数－信号強度特性を図４に示す。横軸はベースバンド周波数、縦軸は受光素子２２，１５の出力信号強度である。受光素子２２，１５は受光強度に比例した信号を出力し、その出力信号はベースバンド上に表れる。強度変調光は、周波数F_pの変調信号で光強度変調がかけられているので、受光素子出力には直流成分と周波数F_pの成分が表れる。ここで、光増幅器７０の入力段における受光素子２２の出力信号強度はＥinとなり、出力段における受光素子１５の出力信号強度はＥout となり、光増幅器７０の光増幅利得Ｇは、

Ｇ＝Ｅout／Ｅin　　…(2)

と表すことができる。したがって、光増幅器７０の入力光および出力光を各受光素子２２，１５で２乗検波し、中心周波数F_pの帯域通過フィルタ２３，１６を用いて周波数F_pの変調信号強度を測定することにより、光増幅器７０の出力レベルや光増幅利得を制御することができる。なお、光増幅器７０の出力光には増幅された自然放出光雑音（ＡＳＥ）も混入するが、光周波数f_pの近傍の帯域内に存在するＡＳＥ電力は比較的小さく、その影響はほとんどない。

【００３５】なお、以上の説明では、ベースバンド帯における変調信号成分の検出例を示したが、ヘテロダイン検波その他を用いて中間周波数帯上で目的とする変調信号成分を検出することも可能である。

【００３６】次に、入力光信号が光周波数多重化されている場合に、入力光信号の多重数の変動および光増幅利得特性の光周波数依存性の影響を受けない光増幅器の出力制御回路の実施例について説明する。

【００３７】図５は、請求項３に記載の光増幅器の出力制御回路の実施例構成を示す。なお、本実施例は、光増幅器の出力レベルを安定化する出力レベル制御回路としての構成を示す。

【００３８】図において、光信号入力端子７２には光周波数f_1～f_nの光信号が多重化されて入力される。光増幅器７０は、励起光源７１から出力される励起光の作用により、光信号入力端子７２から入力された光周波数多重信号を増幅して光信号出力端子７３に送出する。

【００３９】この光増幅器７０の出力レベルを各光周波数ごとに安定化する本実施例の出力制御回路（出力レベル制御回路）は、所定の光信号源11_i，11_j，11_kから出力される光周波数f_i，f_j，f_kの光信号に対して、強度変調する周波数F_i，F_j，F_kの変調信号を発生する低周波発振器12_i，12_j，12_k、各変調信号で駆動される強度変調器13_i，13_j，１３

$_k$、光多重回路５１、光分岐素子１４、受光素子１５、変調信号の周波数F_i，F_j，F_kを中心周波数とする帯域通過フィルタ（ＢＰＦ）16_i，16_j，16_k、包絡線検波器17_i，17_j，17_k、制御回路５２、光増幅器７０の出力段に挿入される光利得等化回路５３により構成される。

【００４０】なお、低周波発振器12_i，12_j，12_kおよび強度変調器13_i，13_j，13_kは、強度変調光入力手段に対応する。光分岐素子１４および受光素子１５は、受光手段に対応する。帯域通過フィルタ16_i，16_j，16_kおよび包絡線検波器17_i，17_j，17_kは、変調信号強度測定手段に対応する。光利得等化回路５３はそのまま対応し、制御回路５２および励起光源７１は、出力レベル制御手段に対応する。

【００４１】光利得等化回路５３は、光周波数f_i，f_j，f_kの光を合分波する分波器５４および合波器５５、光減衰器（ＯＡＴＴ）56_i，56_j，56_kにより構成される。なお、分波器５４および合波器５５には、グレーティングフィルタまたはアレイ導波路型フィルタその他の光合分波機能を有する光フィルタが用いられる。また、光利得等化回路５３として、透過率の波長依存性を可変させる光フィルタ、例えば音響光学フィルタ，多段縦属接続マハツェンダ型フィルタその他を使用することもできる。

【００４２】各強度変調器13_i，13_j，13_kは、光信号源11_i，11_j，11_kから出力された信号光を低周波発振器12_i，12_j，12_kから出力された変調信号で強度変調し、その強度変調光は他の光信号とともに光多重回路５１で光周波数多重されて光増幅器７０に入力される。

【００４３】光増幅器７０の出力光は、光利得等化回路５３に入力され、各光周波数f_i，f_j，f_kの光強度がそれぞれ調整される。光利得等化回路５３の出力光の一部は、光分岐素子１４で分岐して受光素子１５に受光される。受光素子１５は受光強度に比例する信号を出力し、その信号は各帯域通過フィルタ16_i，16_j，16_kを介してそれぞれ包絡線検波器17_i，17_j，17_kに入力される。各包絡線検波器17_i，17_j，17_kは、光増幅器７０の出力光強度に比例した周波数F_i，F_j，F_kの変調信号電圧を出力する。制御回路５２は、各周波数F_i，F_j，F_kの変調信号電圧から、それぞれに比例した光周波数f_i，f_j，f_kの光強度を検出し、それらを一定するために光利得等化回路５３の各光減衰器56_i，56_j，56_kの減衰率を制御する制御信号I_i，I_j，I_kを算出する。

【００４４】また、制御回路５２は、各周波数F_i，F_j，F_kの変調信号電圧から光利得等化回路５３の出力レベルの変動量を検出し、それを光増幅器７０の励起光源７１に帰還してバイアス電流値を制御することにより、光利得等化回路５３の出力レベルを安定化させる。

【００４５】このような構成により、光増幅器７０の光周波数帯域上に広く分布する自然放出光雑音の影響をほとんど受けずに、各光周波数帯域の出力レベルを個別に制御することができる。また、入力光信号が光周波数多重化されている場合に、入力光信号の多重数が変動しても、また光増幅利得特性の光周波数依存性があっても各光周波数帯域の出力レベルをそれぞれ個別に安定化することができる。

【００４６】図６は、請求項４に記載の光増幅器の出力制御回路の実施例構成を示す。なお、本実施例は、光増幅器の光増幅利得を一定にする利得制御回路としての構成を示す。

【００４７】図において、光信号入力端子７２には光周波数$f_1 \sim f_n$の光信号が多重化されて入力される。光増幅器７０は、励起光源７１から出力される励起光の作用により、光信号入力端子７２から入力された光周波数多重信号を増幅して光信号出力端子７３に送出する。

【００４８】この光増幅器７０の光増幅利得を各光周波数ごとに一定にする本実施例の出力制御回路（利得制御回路）は、図５に示す出力制御回路（出力レベル制御回路）の構成に加えて、光増幅器７０の入力段に設けられる同様の光分岐素子２１、受光素子２２、帯域通過フィルタ（ＢＰＦ）23_i，23_j，23_k、包絡線検波器24_i，24_j，24_kを含む。

【００４９】なお、低周波発振器12_i，12_j，12_kおよび強度変調器13_i，13_j，13_kは、強度変調光入力手段に対応する。光分岐素子２１および受光素子２２は、入力段受光手段に対応する。帯域通過フィルタ23_i，23_j，23_kおよび包絡線検波器24_i，24_j，24_kは、入力段変調信号強度測定手段に対応する。光分岐素子１４および受光素子１５は、出力段受光手段に対応する。帯域通過フィルタ16_i，16_j，16_kおよび包絡線検波器17_i，17_j，17_kは、出力段変調信号強度測定手段に対応する。光利得等化回路５３はそのまま対応し、制御回路５２および励起光源７１は、光増幅利得制御手段に対応する。

【００５０】各強度変調器13_i，13_j，13_kは、光信号源11_i，11_j，11_kから出力された信号光を低周波発振器12_i，12_j，12_kから出力された変調信号で強度変調し、その強度変調光は他の光信号とともに光多重回路５１で光周波数多重されて光増幅器７０に入力される。

【００５１】光増幅器７０の入力光の一部は、光分岐素子２１で分岐して受光素子２２に受光される。受光素子２２は受光強度に比例する信号を出力し、その信号は帯域通過フィルタ23_i，23_j，23_kを介して、それぞれ包絡線検波器24_i，24_j，24_kに入力される。各包絡線検波器24_i，24_j，24_kは、光増幅器７０の入力光強度に比例した周波数F_i，F_j，F_kの変調信号電圧を出力する。

【００５２】光増幅器７０の出力光は、光利得等化回路５３で各光周波数f_i，f_j，f_kの光強度が調整される。光利得等化回路５３の出力光の一部は、光分岐素子１４で分岐して受光素子１５に受光される。受光素子１５は受光強度に比例する信号を出力し、その信号は各帯域通過フィルタ16_i，16_j，16_kを介してそれぞれ包絡線検波器17_i，17_j，17_kに入力される。各包絡線検波器17_i，17_j，17_kは、光増幅器７０の出力光強度に比例した周波数F_i，F_j，F_kの変調信号電圧を出力する。

【００５３】制御回路５２は、光増幅器７０の入力光強度および光利得等化回路５３の出力光強度に比例した各周波数F_i，F_j，F_kの変調信号電圧を比較し、光周波数f_i，f_j，f_kの各光信号に対する光増幅利得を算出する。ここで、各光増幅利得が規定値からずれている場合には、光利得等化回路５３を制御して利得の不均一を調整する。また、利得の過不足が生じた場合には、光増幅器７０の励起光源７１のバイアス電流値を制御し、光増幅器７０の光増幅利得を制御して調整する。

【００５４】このような構成により、光増幅器７０の光周波数帯域上に広く分布する自然放出光雑音の影響をほとんど受けずに、各光周波数帯域の光増幅利得を個別に制御することができる。また、入力光信号が光周波数多重化されている場合に、入力光信号の多重数が変動しても、また光増幅利得特性の光周波数依存性があっても各光周波数帯域の光増幅利得をそれぞれ個別に安定化することができる。

【００５５】

【発明の効果】以上説明したように、本発明の光増幅器の出力制御回路は、光増幅器の出力光に含まれる自然放出光雑音の影響を受けずに、出力レベルあるいは光増幅利得を安定化制御することができる。

【００５６】また、光周波数多重化された光信号が入力される場合でも、入力光信号の多重数の変動および光増幅利得特性の光周波数依存性の影響を受けずに、出力レベルあるいは光増幅利得を安定化制御することができる。

【図面の簡単な説明】

【図１】請求項１に記載の光増幅器の出力制御回路の実施例構成を示すブロック図。

【図２】請求項２に記載の光増幅器の出力制御回路の実施例構成を示すブロック図。

【図３】光増幅器７０における強度変調光の増幅を説明する図。

【図４】受光素子２２，１５の出力信号の周波数－信号強度特性を示す図。

【図５】請求項３に記載の光増幅器の出力制御回路の実施例構成を示すブロック図。

【図６】請求項４に記載の光増幅器の出力制御回路の実施例構成を示すブロック図。

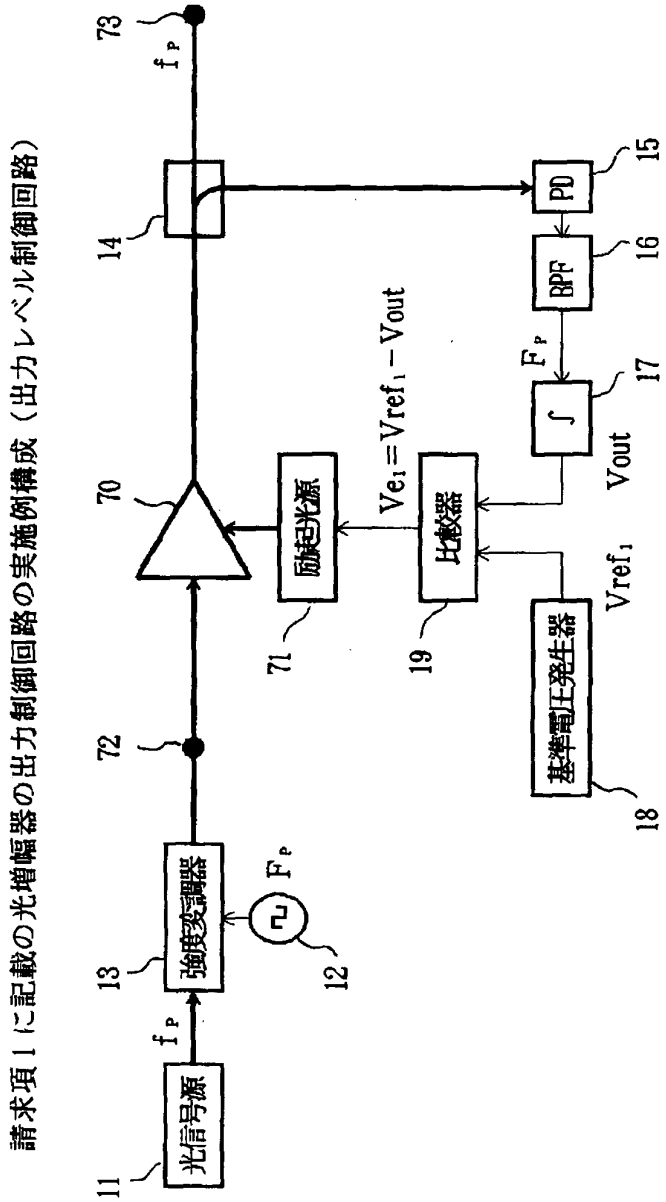
【図７】従来の光増幅器の出力レベル制御回路の構成例を示すブロック図。

【図８】励起光源７１から出力される励起光強度と光増幅器７０の出力光強度の関係を示す図。

【符号の説明】

１１　光信号源
１２　低周波発振器
１３　強度変調器
１４，２１，７４　光分岐素子
１５，２２，７５　受光素子（ＰＤ）
１６，２３　帯域通過フィルタ（ＢＰＦ）
１７，２４　包絡線検波器
１８，７８　基準電圧発生器
１９，７７　比較器
２５　除算器
５１　光多重回路
５２　制御回路
５３　光利得等化回路
５４　分波器
５５　合波器
５６　光減衰器（ＯＡＴＴ）
７０　光増幅器
７１　励起光源
７２　光信号入力端子
７３　光信号出力端子
７６　増幅器
７９　低域通過フィルタ（ＬＰＦ）

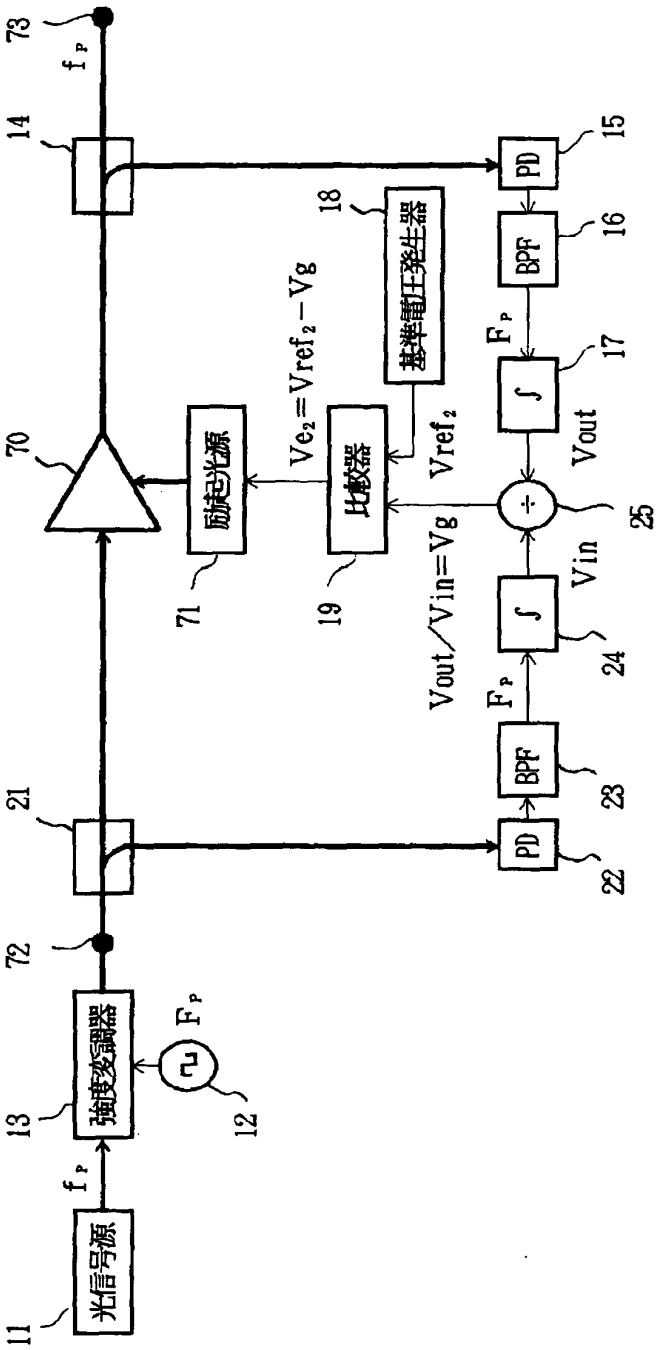
【図1】



請求項１に記載の光増幅器の出力制御回路の実施例構成（出力レベル制御回路）

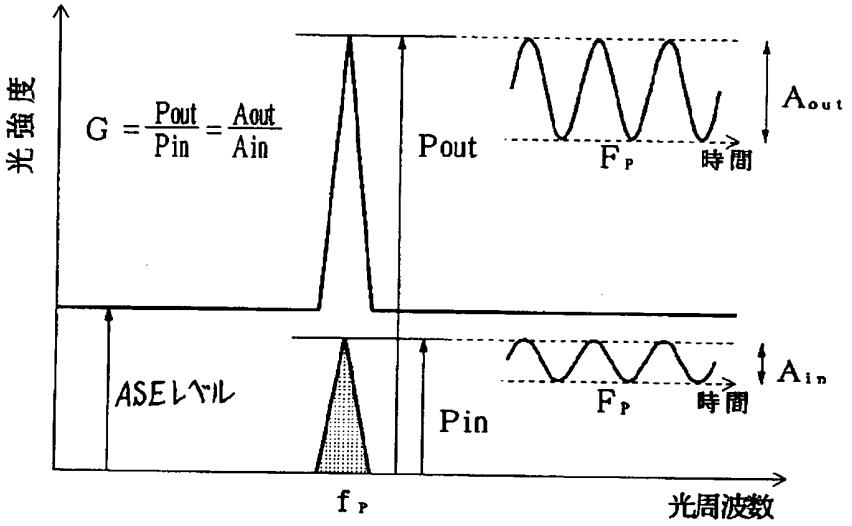
【図2】

請求項２に記載の光増幅器の出力制御回路の実施例構成（利得制御回路）



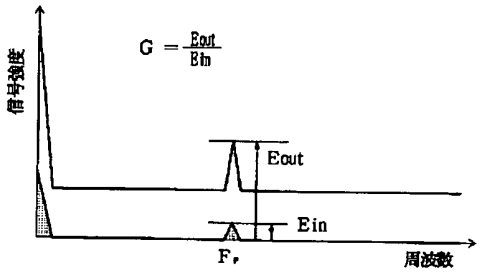
【図３】

光増幅器70における強度変調光の増幅例



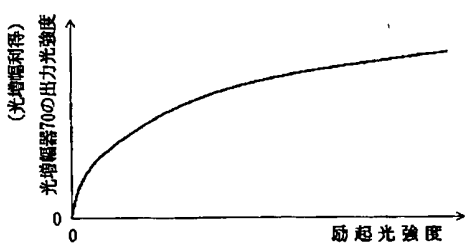
【図４】

受光素子22，15の出力信号の周波数－信号強度特性



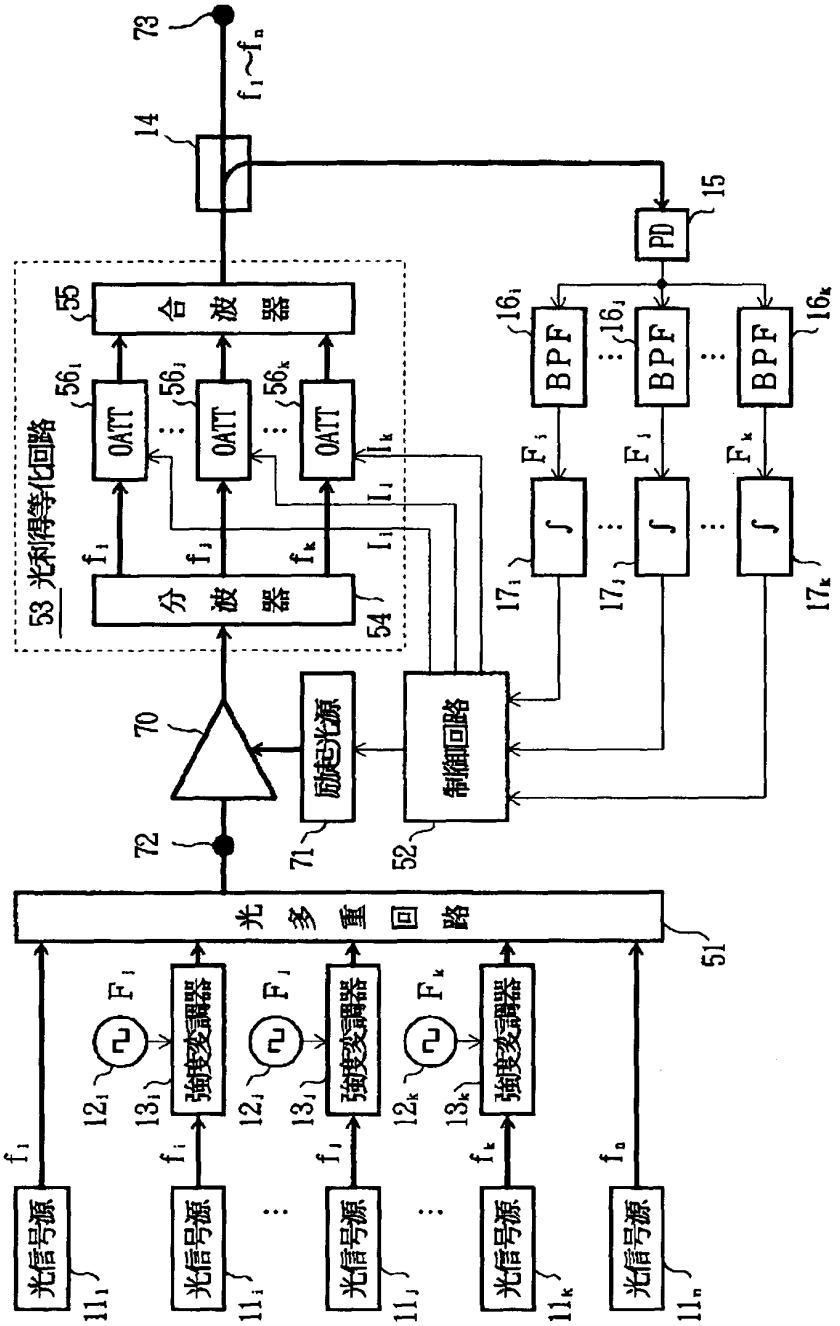
【図８】

励起光強度と光増幅器70の出力光強度の関係



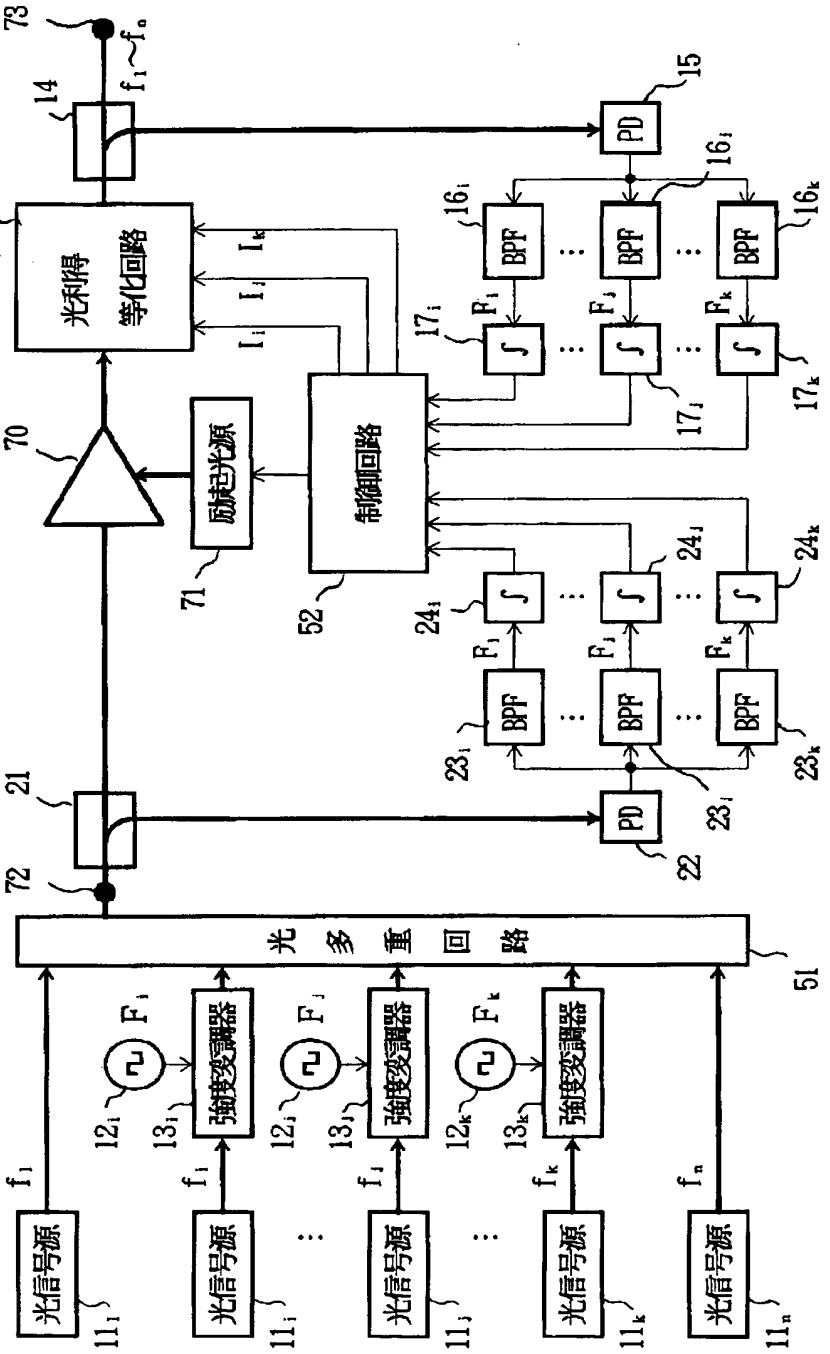
【図5】

請求項3に記載の光増幅器の出力制御回路の実施例構成（出力レベル制御回路）



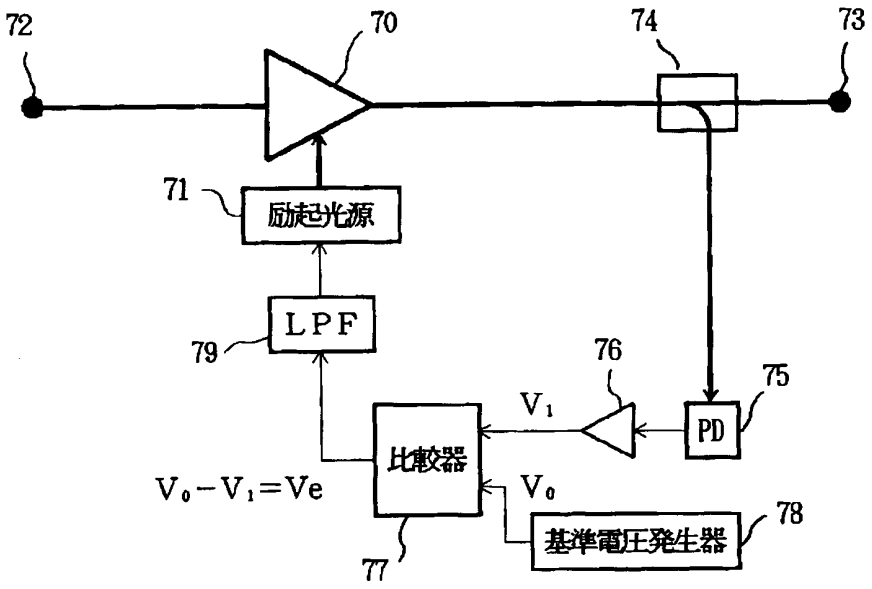
【図6】

請求項4に記載の光増幅器の出力制御回路の実施例構成（利得制御回路）



【図7】

従来の光増幅器の出力レベル制御回路の構成例



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(22)Date of filing : 23.07.1992

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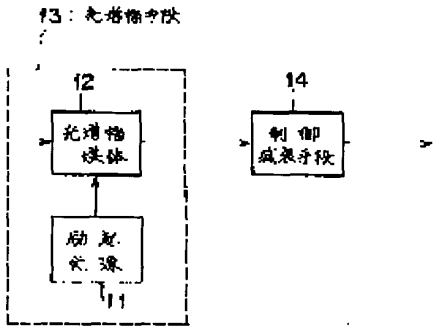
(72)Inventor : NISHIMOTO HIROSHI

(54) OPTICAL AMPLIFIER

(57)Abstract:
PURPOSE: To provide an optical amplifier in which power of an output signal light can be held constant irrespective of an abrupt change of power of an input signal light.
CONSTITUTION: The optical amplifier comprises optical amplifying means 13 having an exciting light source 11 and an optical amplifying medium 12 in which an exciting light from the source 11 is guided, and control attenuating means 14 for attenuating the output of the means 13 by an attenuation factor so controlled that an output power becomes constant.



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(54)【発明の名称】　光増幅器

(57)【要約】
【目的】本発明は光増幅器に関し、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができる光増幅器の提供を目的とする。
【構成】励起光源１１と信号光及び励起光源１１からの励起光が導波される光増幅媒体１２とを含む光増幅手段１３と、光増幅手段１３の光出力を、出力パワーが一定になるように制御された減衰率で減衰させる制御減衰手段１４とから構成する。

【特許請求の範囲】

【請求項１】　励起光源(11)と信号光及び該励起光源(11)からの励起光が導波される光増幅媒体(12)とを含む光増幅手段(13)と、
該光増幅手段(13)の光出力を、出力パワーが一定になるように制御された減衰率で減衰させる制御減衰手段(14)とを備えたことを特徴とする光増幅器。

【請求項２】　上記光増幅手段(13)の光出力を第１分岐光及び第２分岐光に分岐する第１の光分岐手段(31)と、
上記第１分岐光のパワーに基づき上記励起光のパワーをフィードバック制御する励起光制御手段(32)とをさらに備え、
上記第２分岐光が上記制御減衰手段(14)に供給されることを特徴とする請求項１に記載の光増幅器。

【請求項３】　上記制御減衰手段(14)は、上記第２分岐光が供給される減衰率可変型の光減衰器(33)を含むことを特徴とする請求項２に記載の光増幅器。

【請求項４】　上記制御減衰手段(14)は、上記光減衰器(33)の光出力を第３分岐光及び第４分岐光に分岐する第２の光分岐手段(34)と、上記第３分岐光のパワーに基づき上記光減衰器(33)の減衰率をフィードバック制御する減衰率フィードバック制御手段(35)とをさらに含み、
該減衰率フィードバック制御手段(35)における制御による光パワーの変化の応答速度が上記励起光制御手段(32)における制御による光パワーの変化の応答速度よりも十分に速いことを特徴とする請求項３に記載の光増幅器。

【請求項５】　上記励起光源(11)はレーザダイオード(51)からなり、
上記励起光制御手段(32)は、上記第１分岐光が入力され該第１分岐光の平均パワーに応じた直流電圧信号を出力する第１の受光回路(58)と、該第１の受光回路(58)からの直流電圧信号を第１の参照電圧と比較してその誤差成分が零になるように上記レーザダイオード(51)のバイアス電流を制御するバイアス制御回路(59)とを含み、
上記減衰率フィードバック制御手段(35)は、上記第３分岐光が入力され該第３分岐光の平均パワーに応じた直流電圧信号を出力する第２の受光回路(60)と、該第２の受光回路(60)からの直流電圧信号を第２の参照電圧と比較してその誤差成分が零になるように上記光減衰器(33)の減衰率を制御する減衰率制御回路(61)とを含むことを特徴とする請求項４に記載の光増幅器。

【請求項６】　上記第２の受光回路(60)からの直流電圧信号が上記第２の参照電圧に等しいとしたときの上記光減衰器(33)の光出力のパワーは、上記第１の受光回路(58)からの直流電圧信号が上記第１の参照電圧に等しいとしたときの上記光減衰器(33)の光入力のパワーに等しいかそれよりも小さいことを特徴とする請求項５に記載の光増幅器。

【請求項７】　上記制御減衰手段(14)は、上記第１分岐光のパワーに基づき上記光減衰器(33)の減衰率をフィードフォワード制御する減衰率フィードフォワード制御手段(41)をさらに含み、
該減衰率フィードフォワード制御手段(41)における制御による光パワーの変化の応答速度が上記励起光制御手段(32)における制御による光パワーの変化の応答速度よりも十分に速いことを特徴とする請求項３に記載の光増幅器。

【請求項８】　上記光減衰器(33)に入力する上記第２分岐光を遅延させる光遅延手段をさらに備え、
該光遅延手段における遅延時間は上記減衰率フィードフォワード制御手段(41)の制御応答特性に応じて設定されることを特徴とする請求項７に記載の光増幅器。

【請求項９】　上記励起光源(11)はレーザダイオード(51)からなり、
上記励起光制御手段(32)は、上記第１分岐光が入力され該第１分岐光の平均パワーに応じた直流電圧信号を出力する受光回路(58)と、該受光回路(58)からの直流電圧信号を第１の参照電圧と比較してその誤差成分が零になるように上記レーザダイオード(51)のバイアス電流を制御するバイアス制御回路(59)とを含み、
上記減衰率フィードフォワード制御手段(41)は、上記受光回路(58)からの直流電圧信号を第２の参照電圧と比較してその誤差成分が零になるように上記光減衰器(33)の減衰率を制御する減衰率制御回路(101) を含むことを特徴とする請求項８に記載の光増幅器。

【請求項１０】　上記受光回路(58)からの直流電圧信号が上記第２の参照電圧に等しいとしたときの上記光減衰器(33)の光出力のパワーは、上記受光回路(58)からの直流電圧信号が上記第１の参照電圧に等しいとしたときの上記光減衰器(33)の光入力のパワーに等しいかそれよりも小さいことを特徴とする請求項９に記載の光増幅器。

【請求項１１】　上記光減衰器(33)は、$LiNbO_3$ マッハツェンダ型光変調器、半導体電界吸収型光変調器及び半導体マッハツェンダ型光変調器から選択される光変調器からなり、該光変調器に与えられる電圧信号により上記減衰率が制御されることを特徴とする請求項３に記載の光増幅器。

【請求項１２】　上記制御減衰手段(14)は、上記第２分岐光を第１偏波成分と該第１偏波成分の偏波面に垂直な偏波面を有する第２偏波成分に偏波分離する偏波分離手段と、上記第１偏波成分が入力する減衰率可変型の第１の光減衰器(92A) と、上記第２偏波成分が入力する減衰率可変型の第２の光減衰器(92B) と、該第１及び第２の光減衰器からそれぞれ出力される偏波成分を偏波合成する偏波合成手段とを含むことを特徴とする請求項２に記載の光増幅器。

【請求項１３】　上記励起光源(11)はレーザダイオード(51)からなり、
上記光増幅媒体(12)は少なくともコアに希土類元素がドープされたドープファイバ(52)からなり、

上記光増幅手段(13)は、
上記信号光及び上記励起光がそれぞれ入力する第１ポート(53A) 及び第２ポート(53B) 並びに合波された上記信号光及び上記励起光が出力する第３ポート(53C) を有する光合波器(53)と、
入力ポート(54A) 及び出力ポート(54B) を有し、該入力ポート(54A) は上記光合波器(53)の第３ポート(53C) に接続され、該出力ポート(54B) は上記ドープファイバ(52)の第１端に接続される第１の光アイソレータ(54)と、
入力ポート(55A) 及び出力ポート(55B) を有し、該入力ポート(55A) は上記ドープファイバ(52)の第２端に接続される第２の光アイソレータ(55)と、
入力ポート(56A) 及び出力ポート(56B) を有し、該入力ポート(56A) は上記第２の光アイソレータ(55)の出力ポート(55B) に接続され、その出力ポート(56B)の光出力が上記制御減衰手段に供給される光帯域通過フィルタ(56)とをさらに含むことを特徴とする請求項１に記載の光増幅器。

【請求項１４】　上記信号光の波長は１．５５μm帯にあり、上記励起光の波長は１．４８μm帯又は０．９８μm帯にあり、上記ドープファイバ(52)にドープされる希土類元素はＥｒ（エルビウム）であり、上記光帯域通過フィルタ(56)の通過波長帯域は１．５５μm帯にあることを特徴とする請求項１３に記載の光増幅器。

【請求項１５】　励起光源(11)と信号光及び該励起光源(11)からの励起光が導波される光増幅媒体(12)とを含む光増幅手段(13)と、
該光増幅手段(13)の光出力を第１分岐光及び第２分岐光に分岐する光分岐手段(21)と、
上記光増幅媒体(12)で誘導放出を生じさせる波長帯の制御光を上記光増幅媒体(12)に上記信号光の伝搬方向下流側から導入する制御光導入手段(22)と、
上記第１分岐光のパワーに基づき上記制御光のパワーをフィードバック制御する制御光制御手段(23)とを備えたことを特徴とする光増幅器。

【請求項１６】　上記第１分岐光のパワーに基づき上記励起光のパワーをフィードバック制御する励起光制御手段をさらに備え、
上記制御光制御手段(23)における制御による光パワーの変化の応答速度が上記励起光制御手段における制御による光パワーの変化の応答速度よりも十分速いことを特徴とする請求項１５に記載の光増幅器。

【請求項１７】　上記励起光源(11)は第１のレーザダイオード(51)からなり、
上記光増幅媒体(12)は少なくともコアに希土類元素がドープされたドープファイバ(52)からなり、
上記光増幅手段(13)は、
上記信号光及び上記励起光がそれぞれ入力する第１ポート(53A) 及び第２ポート(53B) 並びに合波された上記信号光及び上記励起光が出力する第３ポート(53C) を有する光合波器(53)と、
入力ポート(54A) 及び出力ポート(54B) を有し、該入力ポート(54A) は上記光合波器(53)の第３ポート(53C) に接続され、該出力ポート(54B) は上記ドープファイバ(52)の第１端に接続される光アイソレータ(54)と、
入力ポート(56A) 及び出力ポート(56B) を有し、該入力ポート(56A) は上記ドープファイバ(52)の第２端に接続され、該出力ポート(56B) は上記光分岐手段に接続される光帯域通過フィルタ(56)とをさらに含み、
上記励起光制御手段は、
上記第１分岐光が入力され該第１分岐光の平均パワーに応じた直流電圧信号を出力する受光回路(58)と、
該受光回路(58)からの直流電圧信号を第１の参照電圧と比較してその誤差成分が零になるように上記第１のレーザダイオード(51)のバイアス電流を制御する第１のバイアス制御回路(59)とを含み、
上記制御光導入手段(22)は、
上記制御光を出力する第２のレーザダイオード(112)と、
該第２のレーザダイオード(112) に接続される第１ポート(111A)と上記光分岐手段に接続される第２ポート(111B)と出力側光伝送路に接続される第３ポート(111C)とデッドエンドにされる第４ポート(111D)とを有し、該第１ポート(111A)に入力した光を該第２ポート(111B)のみから出力し、該第２ポート(111B)に入力した光を該第３ポート(111C)のみから出力し、該第３ポート(111C)に入力した光を該第４ポート(111D)のみから出力し、該第４ポート(111D)に入力した光を該第１ポート(111A)のみから出力する光サーキュレータ(111) とを含み、
上記制御光制御手段(23)は、上記受光回路(58)からの直流電圧信号を第２の参照電圧と比較してその誤差成分が零になるように上記第２のレーザダイオード(112) のバイアス電流を制御する第２のバイアス制御回路(113) を含むことを特徴とする請求項１６に記載の光増幅器。

【請求項１８】　上記受光回路(58)からの直流電圧信号が上記第２の参照電圧に等しいとしたときの上記第２分岐光のパワーは、上記受光回路(58)からの直流電圧信号が上記第１の参照電圧に等しいとしたときの上記第２分岐光のパワーに等しいかそれよりも大きいことを特徴とする請求項１７に記載の光増幅器。

【請求項１９】　上記制御光の波長は上記信号光の波長にほぼ等しいことを特徴とする請求項１５に記載の光増幅器。

【発明の詳細な説明】

【０００１】

(目次)

作用
実施例
発明の効果
【０００２】
【産業上の利用分野】本発明は光増幅器に関する。光増幅器の光伝送システムへの適用形態としては、光中継器、光送信機用の電力増幅器、光受信機用の前置増幅器等がある。このような光増幅器の用途においては、入力した光のパワーにかかわらず出力される光のパワーが一定になるような制御を行うことが、後段の装置の安定動作を確保する上で要求される。
【０００３】
【従来の技術】励起光により光増幅媒体を励起状態にし、光増幅媒体内で生じる誘導放出により信号光を増幅するように構成された光増幅器が公知である。
【０００４】この種の光増幅器においては、一般に、出力される信号光のパワーが一定になるようにするために、光増幅媒体の光出力を分岐し、分岐光のパワーに基づき励起光のパワーを制御するフィードバックループが付加的に設けられている。
【０００５】光増幅媒体としては、例えば、Ｅｒ（エルビウム）等の希土類元素がドープされたドープファイバが用いられる。
【０００６】
【発明が解決しようとする課題】光増幅媒体としてドープファイバが用いられている場合、上述のようなフィードバックループを設けたとしても、入力信号光のパワーが急峻に変化したときに、励起光のパワーの変化に対する光増幅媒体の応答が遅いために、出力信号光のパワーが必ずしも一定に保たれないことがあるという問題が生じる。
【０００７】例えば、入力信号光のパワーが急激に増大した場合、フィードバックループにより励起光のパワーは急激に減少させられるが、励起光のパワーの減少に伴う光増幅媒体の反転分布の減少は急激にはなされない（ドープ元素がＥｒである場合十数ミリ秒必要）ので、出力信号光はサージ状のパワー変動を伴うことになる。また、入力信号光のパワーが急激に減少した場合に制御のオーバーシュートによりサージ状の極端なパワー変動が生じることもある。このようなサージ状のパワー変動が生じると、例えば光受信機における受光素子やトランジスタが破損する恐れが生じる。
【０００８】本発明の目的は、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができる光増幅器を提供することにある。
【０００９】
【課題を解決するための手段】図１は本発明の光増幅器の第１構成を示すブロック図であり、この構成は請求項１により特定される構成に対応している。
【００１０】本発明の光増幅器の第１構成は、励起光源１１と信号光及び励起光源１１からの励起光が導波される光増幅媒体１２とを含む光増幅手段１３と、光増幅手段１３の光出力を、出力パワーが一定になるように制御された減衰率で減衰させる制御減衰手段１４とを備える。
【００１１】図２は本発明の光増幅器の第２構成を示すブロック図であり、この構成は請求項１５により特定される構成に対応している。本発明の光増幅器の第２構成は、励起光源１１と信号光及び励起光源１１からの励起光が導波される光増幅媒体１２とを含む光増幅手段１３と、光増幅手段１３の光出力を第１分岐光及び第２分岐光に分岐する光分岐手段２１と、光増幅媒体１２で誘導放出を生じさせる波長帯の制御光を光増幅媒体１２に上記信号光の伝搬方向下流側から導入する制御光導入手段２２と、上記第１分岐光のパワーに基づき上記制御光のパワーをフィードバック制御する制御光制御手段２３とを備える。
【００１２】
【作用】本発明の光増幅器の第１構成によると、出力パワーが一定になるように減衰率が制御される制御減衰手段を設けており、この種の減衰率制御では制御の応答速度を十分に速くすることができるので、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができる光増幅器の提供が可能になる。
【００１３】本発明の光増幅器の第２構成において、光増幅媒体１２で誘導放出を生じさせる波長帯の制御光が、制御光導入手段２２により光増幅媒体１２に信号光の伝搬方向下流側から導入されると、光増幅媒体１２における反転分布は導入された制御光のパワーに応じて減少させられる。反転分布が減少すると、光増幅手段１３における利得は下がる。この場合、光増幅媒体１２における利得の減少は、制御光のパワーの変化（増大）に高速で応答する。
【００１４】従って、光分岐手段２１からの分岐光のパワーに基づき制御光のパワーをフィードバック制御することによって、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができるようになる。
【００１５】
【実施例】以下、本発明の実施例を説明する。尚、全図を通して実質的に同一の部分には同一の符号を付すことにする。
【００１６】図３は本発明の光増幅器の第１構成の第１実施態様を示すブロック図である。この第１実施態様においては、光増幅手段１３の光出力を第１分岐光及び第２分岐光に分岐する光分岐手段３１と、光分岐手段３１からの第１分岐光のパワーに基づき励起光のパワーをフィードバック制御する励起光制御手段３２とが付加的に設けられており、光分岐手段３１からの第２分岐光が制御減衰手段（図１参照）に供給される。

【００１７】制御減衰手段は、光分岐手段３１からの第２分岐光が供給される減衰率可変型の光減衰器３３と、光減衰器３３の光出力を第３分岐光及び第４分岐光に分岐する光分岐手段３４と、光分岐手段３４からの第３分岐光のパワーに基づき光減衰器３３の減衰率をフィードバック制御する減衰率フィードバック制御手段３５とを含む。

【００１８】減衰率フィードバック制御手段３５における制御による光パワー（光減衰器３３の光出力のパワー）の変化の応答速度は、励起光制御手段３２における制御による光パワー（光増幅媒体１２の光出力のパワー）の変化の応答速度よりも十分に速い。

【００１９】光分岐手段３１及び３４における分岐比は、第１及び第３分岐光の必要強度に応じて設定される。第１及び第２分岐光間の分岐比並びに第３及び第４分岐光間の分岐比は、例えば、それぞれ１：２０である。

【００２０】図４は本発明の光増幅器の第１構成の第２実施態様を示すブロック図である。第２実施態様は、光分岐手段３１及び励起光制御手段３２が付加的に設けられている点並びに制御減衰手段（図１参照）が光減衰器３３を含む点で図３の第１実施態様と共通する。図４の第２実施態様が図３の第１実施態様と異なる点は、光減衰器３３の減衰率をフィードフォワード制御するようにしている点である。

【００２１】即ち、第２実施態様においては、制御減衰手段（図１参照）は、光減衰器３３に加えて、光分岐手段３１からの第１分岐光のパワーに基づき光減衰器３３の減衰率をフィードフォワード制御する減衰率フィードフォワード制御手段４１を含む。

【００２２】減衰率フィードフォワード制御手段４１における制御による光パワー（光減衰器３３の光出力のパワー）の変化の応答速度は、励起光制御手段３２における制御による光パワー（光増幅媒体１２の光出力のパワー）の変化の応答速度よりも十分に速い。

【００２３】尚、減衰率フィードフォワード制御手段４１の制御応答特性によっては、入力信号光のパワーの急峻な変化に光減衰器３３の減衰率の変化が追従し得ない恐れがあるので、このような場合には、遅延時間が減衰率フィードフォワード制御手段４１の制御応答特性に応じて設定される光遅延回路を光分岐手段３１と光減衰器３３の間に設けるとよい。

【００２４】図５は図３の第１実施態様の具体的実施例を示す光増幅器のブロック図である。図３の光増幅手段１３は、励起光源１１に対応するレーザダイオード５１と、光増幅媒体１２に対応するドープファイバ５２と、光合波器５３と、光アイソレータ５４及び５５と、光帯域通過フィルタ５６とを含む。

【００２５】図３の光分岐手段３１及び３４に対応して、それぞれ光分岐回路５７及び５９が設けられている。光分岐回路５７及び５９は、例えば光カプラ等の光方向性結合器からなる。

【００２６】図３の励起光制御手段３２は、受光回路５８及びバイアス制御回路５９を含む。図３の減衰率フィードバック制御手段３５は、受光回路６０及び減衰率制御回路６１を含む。

【００２７】光合波器５３のポート５３Ａには増幅すべき信号光が供給され、ポート５３Ｂにはレーザダイオード５１からの励起光が供給される。信号光及び励起光は光合波器５３で合波され、ポート５３Ｃから出力される。

【００２８】ドープファイバ５２の少なくともコアにはＥｒ（エルビウム），Ｎｄ（ネオジム），Ｐｒ（プラセオジム）等の希土類元素がドープされている。ドープ元素の種類及び励起光の波長は、増幅すべき信号光の波長に応じて設定される。信号光の波長が石英系光ファイバにおける損失特性が良好な１．５５μｍ帯にある場合において、ドープ元素がＥｒであるときには、励起光の波長は１．４８μｍ帯或いは０．９８μｍ帯に設定される。

【００２９】光合波器５３のポート５３Ｃは、光アイソレータ５４の入力ポート５４Ａに接続され、光アイソレータ５４の出力ポート５４Ｂはドープファイバ５２の第１端に接続される。

【００３０】ドープファイバ５２の第２端は光アイソレータ５５の入力ポート５５Ａに接続され、光アイソレータ５５の出力ポート５５Ｂは光帯域通過フィルタ５６の入力ポート５６Ａに接続される。

【００３１】光アイソレータ５４及び５５はそれぞれ入力ポートから出力ポートに向かう順方向にのみ光を通過させる。光分岐回路５７は、ポート５７Ａに供給された光を所定の分岐比（例えば２０：１）で分岐してそれぞれポート５７Ｂ及び５７Ｃから出力する。光合波器５７のポート５７Ｂ及び５７Ｃはそれぞれ光減衰器３３及び受光回路５８に接続される。

【００３２】光分岐回路５９は、光減衰器３３の光出力を２分岐して、一方の分岐光を受光回路６０に供給する。光分岐回路５９の他方の分岐光はこの光増幅器の光出力となる。光分岐回路５９の分岐比は例えば光分岐回路５７の分岐比と同じである。

【００３３】受光回路５８は、フォトダイオード等の受光素子と増幅器を備えており、この受光回路５８は、光分岐回路５７からの分岐光の平均パワーに応じた直流電圧信号を出力する。

【００３４】バイアス制御回路５９は、受光回路５８からの直流電圧信号を参照電圧V_{ref1}と比較してその誤差成分が零になるようにレーザダイオード５１のバイアス電流を制御する。

【００３５】光減衰器３３としては、$LiNbO_3$ マッハツェンダ型光変調器、半導体電界吸収型光変調器及び

半導体マッハツェンダ型光変調器等の光変調器を用いることができる。ここで例示された光変調器を用いた場合、光変調器に与える電圧信号によりその透過率（即ち減衰率）を高速に制御することができる。

【００３６】受光回路６０は、フォトダイオード等の受光素子と増幅器を備えており、この受光回路６０は、光分岐回路５９からの分岐光の平均パワーに応じた直流電圧信号を出力する。

【００３７】減衰率制御回路６１は、受光回路６０からの直流電圧信号を参照電圧V_{ref2}と比較してその誤差成分が零になるように光減衰器３３の減衰率を制御する。次に、図５の光増幅器の動作を説明する。光合波器５３で合波された信号光及び励起光は、光アイソレータ５４を順方向に通過して、ドープファイバ５２に入射する。ドープファイバ５２に信号光及び励起光が入射すると、導波領域にドープされている希土類元素及び励起光の作用によって誘導放出が生じ、信号光は増幅される。増幅された信号光及び消費されずに残った励起光は、光アイソレータ５５を順方向に通過して光帯域通過フィルタ５６に入射する。光帯域通過フィルタ５６では、励起光及び雑音成分となる自然放出光が除去されて、増幅された信号光のみが光帯域通過フィルタ５６を通過する。

【００３８】励起光のパワーの適当な可変範囲においては、励起光のパワーが増大するのに従って光増幅の利得は増大する。従って、ドープファイバ５２に入力する信号光のパワーが比較的ゆっくりと変動する場合には、受光回路５８及びバイアス制御回路５９の作用によって、信号光のパワー変動を打ち消すようにレーザダイオード５１のバイアス電流が制御される。

【００３９】しかし、ドープファイバ５２に入力する信号光のパワーが急激に変動した場合には、レーザダイオード５１のバイアス電流の制御だけではこのパワー変動は打ち消されず、光減衰器３３に入力する信号光にはパワー変動が残る。そして、このパワー変動が打ち消されるように、光減衰器３３の減衰率が受光回路６０及び減衰率制御回路６１によって制御される。具体的には次の通りである。

【００４０】図６は図５の光減衰器３３に入力する信号光のパワー変化の説明図である。いま、ドープファイバ５２への入力信号光のパワーが、符号７１で表されるように、ステップ応答的に急激に増大した場合を想定する。このようなパワー変動は、光コネクタの着脱、光可変減衰器の操作、何らかの原因による光送信機出力電力の変化等により生じ得るものである。

【００４１】このような入力信号光のパワー変動は受光回路５８により検出され、バイアス制御回路５９はレーザダイオード５１のバイアス電流を即座に減少させて、励起光のパワーは、符号７２で表されるように、入力信号光のパワーの増大とほぼ同時にステップ応答的に減少する。

【００４２】しかし、励起光のパワーが急速に減少させられたとしても、ドープファイバ５２における反転分布量は、符号７３で表されるように、励起光のパワーが減少し始めた時点から比較的ゆっくりと減少する。反転分布量が変化し終えるには、ドープ元素がＥｒである場合には、１０ミリ秒以上の時間を要する。

【００４３】このため、光減衰器３３への入力信号光のパワーは、符号７４で表されるように、サージ状に変化することとなる。このような信号光のパワー変動がそのまま受信側にまで維持されていると、受光素子等に悪影響があることは前述した通りである。

【００４４】図７は図５の光減衰器３３の動作特性の例を示すグラフである。縦軸は透過率Ｔを表し、横軸は制御電圧（例えばバイアス電圧）V_Bを表す。光減衰器３３が$LiNbO_3$マッハツェンダ型光変調器（ＬＮＭＺ光変調器）である場合、透過率Ｔは、符号８１で表されるように、バイアス電圧V_Bの増大に従って正弦波状に周期的に変化する。ＬＮＭＺ光変調器としては、数ＧＨｚ以上の変調信号用のものが実用化されているから、バイアス電圧の変化に対して透過率は極めて高速に応答する。従って、例えば符号８２で表されるように、透過率とバイアス電圧が一対一で対応する領域でバイアス電圧V_Bを変化させることにより、光減衰器３３の減衰率を高速に制御することができる。

【００４５】図５の実施例においては、このような高速な応答特性を有する光減衰器３３を用いて、その減衰率を受光回路６０及び減衰率制御回路６１により制御するようにしているので、図６で符号７４で表されるような光減衰器３３への入力信号光へのパワーの変動があったとしても、これを打ち消すようなフィードバック制御が実現される。

【００４６】図５の実施例では、レーザダイオード５１からの励起光を信号光伝搬方向上流側からドープファイバ５２に供給しているが、励起光を信号光伝搬方向下流側からドープファイバ５２に供給するようにしてもよい。また、信号光の伝搬方向上流側及び下流側の双方からドープファイバ５２に励起光を供給するようにしてもよい。

【００４７】この実施例では、ドープファイバ５２の信号光伝搬方向上流側及び下流側の双方に光アイソレータ５４及び５５をそれぞれ設けているので、共振光路内に光増幅媒体を含む光共振器構造が構成されることが防止され、信号光の安定した増幅が可能になる。

【００４８】尚、図５の実施例において、バイアス制御回路５９及び減衰率制御回路６１における参照電圧V_{ref1}及びV_{ref2}並びに受光回路５８及び６０における光電変換効率並びに光分岐回路５７及び５９の分岐比は、減衰率制御回路６１において受光回路６０からの直流電圧信号が参照電圧V_{ref2}に等しいとしたときの光減衰器３３の光出力のパワー（出力パワー目標値）が、バイア

ス制御回路５９において受光回路５８からの直流電圧信号が参照電圧V_{ref1}に等しいときしたときの光減衰器３３の光入力のパワー（入力パワー目標値）に等しいかそれよりも小さくなるように設定される。

【００４９】接続等による不可避的な損失を考慮すれば、出力パワー目標値が入力パワー目標値よりも小さくなるように上記設定がなされる。何故ならば、出力パワー目標値が入力パワー目標値よりも大きくなるように上記設定がなされると、光減衰器３３においては利得が生じ得ないから、定常状態において減衰率制御回路６１における誤差信号が零にはならず、光減衰器３３が図７に示されたような周期的な動作特性を有している場合に、制御方法によっては制御の暴走が生じる恐れがあるからである。

【００５０】ところで、一般に、光増幅器に入力する光の偏光状態は一定でないため、実用化されているＬＮＭＺ光変調器等のように透過率（減衰率）に偏光依存性がある光減衰器を使用する場合、偏波制御回路等、入力光の偏光の変化に対応する方策を施す必要がある。また、入力光の偏光が一定の場合でも、各光学要素間の接続に用いる光ファイバやドープファイバとして偏波保持ファイバを使用することが要求され、ドープファイバの製造や光増幅器の組立に煩雑な作業が要求される。このような問題を解決し得る実施例を図８により説明する。

【００５１】図８は図３の第１実施態様の他の具体的実施例を示す光増幅器のブロック図である。この実施例では、図３における光増幅手段１３と光分岐手段３１及び３４と励起光制御手段３２と減衰率フィードバック制御手段３５の一部とに対応する部分については、図５の実施例と同じように構成される。

【００５２】光分岐回路５７のポート５７Ｂからの分岐光は、偏光ビームスプリッタ等からなる偏波分離器９１で第１偏波成分と第２偏波成分に偏波分離され、第１及び第２偏波成分はそれぞれ光減衰器９２Ａ及び９２Ｂに入力する。第１偏波成分の偏波面と第２偏波成分の偏波面は互いに垂直である。

【００５３】光減衰器９２Ａ及び９２Ｂは減衰率可変型の同一特性のものであり、各光減衰器９２Ａ及び９２Ｂに入力する偏波成分の偏波面は各光減衰器９２Ａ及び９２Ｂのそれぞれの固有偏波方向（所要の特性が得られるように設定された方向）に一致する。

【００５４】光減衰器９２Ａ及び９２Ｂから出力した各偏波成分は、例えば偏波分離器９１と同じものからなる偏波合成器９３で偏波合成されて、光分岐回路５９に入力する。光分岐回路５９の一方の分岐光は受光回路６０で直流電圧信号に変換される。

【００５５】減衰率制御回路９４は、受光回路６０からの直流電圧信号を参照電圧V_{ref2}と比較してその誤差成分が零になるように光減衰器９２Ａ及び９２Ｂの減衰率を制御する。

【００５６】図５の実施例におけるのと同じ理由により、バイアス制御回路５９及び減衰率制御回路９４における参照電圧V_{ref1}及びV_{ref2}並びに受光回路５８及び６０における光電変換効率並びに光分岐回路５７及び５９の分岐比は、減衰率制御回路９４において受光回路６０からの直流電圧信号が参照電圧V_{ref2}に等しいとしたときの偏波合成器９３の光出力のパワー（出力パワー目標値）が、バイアス制御回路５９において受光回路５８からの直流電圧信号が参照電圧V_{ref1}に等しいとしたときの偏波分離器９１の光入力のパワー（入力パワー目標値）に等しいかそれよりも小さくなるように設定される。光学的な接続等による不可避的な損失を考慮すれば、出力パワー目標値が入力パワー目標値よりも小さくなるように上記設定がなされる。

【００５７】本実施例によると、各光減衰器９２Ａ及び９２Ｂに入力する光の偏波状態は常に一定するので、各光減衰器９２Ａ及び９２Ｂの減衰率に偏波依存性がある場合でも、偏波制御回路等が不要となり、また、各光学要素間の光接続用の光ファイバやドープファイバとして偏波保持ファイバを使用することなしに、本発明の目的を達成することができ、ドープファイバの製造作業や光増幅器の組立作業が簡単になる。

【００５８】図９は図４の第２実施態様の具体的実施例を示す光増幅器のブロック図である。光増幅手段がレーザダイオード５１、ドープファイバ５２、光合波器５３、光アイソレータ５４，５５及び光帯域通過フィルタ５６を含む点と、光分岐手段が光分岐回路５７である点と、励起光制御手段が受光回路５８及びバイアス制御回路５９を含む点と、減衰率可変型の光減衰器３３が用いられている点は図５の実施例と同じである。

【００５９】本実施例では、光分岐回路５７のポート５７Ｂからの分岐光は、所定長さの光ファイバ等からなる光遅延回路１０２を通過した後光減衰器３３に供給され、光減衰器３３の光出力がこの光増幅器から送出される。

【００６０】そして、減衰率制御回路１０１が、受光回路５８からの直流電圧信号を参照電圧V_{ref3}と比較してその誤差成分が零になるように光減衰器３３の減衰率を制御する。

【００６１】光遅延回路１０２における遅延時間は、減衰率制御回路１０１の制御応答特性に応じて設定される。例えば、光遅延回路１０２における遅延時間は、受光回路５８の受光レベルが変化してから光減衰器３３の減衰率が変化し始めるまでの時間にほぼ等しく設定される。

【００６２】図５の実施例におけるのと同じ理由により、バイアス制御回路５９における参照電圧V_{ref1}及び減衰率制御回路１０１における参照電圧V_{ref3}は、減衰率制御回路１０１において受光回路５８からの直流電圧信号が参照電圧V_{ref3}に等しいとしたときの光減衰器３

３の光出力のパワー（出力パワー目標値）が、バイアス制御回路５９において受光回路５８からの直流電圧信号が参照電圧V_{ref1}に等しいとしたときの光減衰器３３の光入力のパワー（入力パワー目標値）に等しいかそれよりも小さくなるように設定される。即ち$V_{ref3} \leqq V_{ref1}$である。接続等による不可避的な損失等を考慮すれば、出力パワー目標値が入力パワー目標値よりも小さくなるように上記設定がなされる。

【００６３】本実施例によると、１つの受光回路５８からの直流電圧信号に基づいてレーザダイオード５１のバイアス電流をフィードバック制御するとともに光減衰器３３の減衰率をフィードフォワード制御するようにしているので、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができ、しかも、図５の実施例に比べて光分岐回路及び受光回路の数を減らして装置の構成を簡単にすることができる。

【００６４】尚、図４の第２実施態様においても、図８の実施例（第１実施態様の具体例）におけるのと同じようにして、光減衰器の偏波依存性に対処してもよい。図１０は図２の本発明の第２構成の具体的実施例を示す光増幅器のブロック図である。本実施例において、光増幅手段の構成が図５の実施例と異なる点は、ドープファイバ５２の信号光伝搬方向下流側の光アイソレータ５５（図５参照）を除去して、ドープファイバ５２の第２端を直接光帯域通過フィルタ５６の入力ポート５６Ａに接続している点である。光分岐手段として光分岐回路５７が用いられている点と、励起光制御手段が受光回路５８及びバイアス制御回路５９を含む点は図５の実施例と同じである。

【００６５】符号１１１はポート１１１Ａ，１１１Ｂ，１１１Ｃ及び１１１Ｄを有する４ポート型の光サーキュレータを表している。ポート１１１Ａに供給された光はポート１１１Ｂのみから出力し、ポート１１１Ｂに供給された光はポート１１１Ｃのみから出力し、ポート１１１Ｃに供給された光はポート１１１Ｄのみから出力し、ポート１１１Ｄに供給された光はポート１１１Ａのみから出力する。

【００６６】ポート１１１Ａにはレーザダイオード１１２からの制御光が供給され、ポート１１１Ｂは光分岐回路５７のポート５７Ｂに接続され、ポート１１１Ｃは図示しない出力側光伝送路に接続され、ポート１１１Ｄはデッドエンドにされる。

【００６７】レーザダイオード１１１からの制御光は、光サーキュレータ１１１、光分岐回路５７及び光帯域通過フィルタ５６をこの順に通過してドープファイバ５２に導入される。また、光分岐回路５７のポート５７Ｂから出力された信号光は、光サーキュレータ１１１を介してこの光増幅器から送出される。

【００６８】このような光サーキュレータ１１１を用いているので、ドープファイバ５２の信号光伝搬方向下流側に光アイソレータを設けなくとも、光増幅媒体を含む光共振器構造が構成される恐れはない。

【００６９】制御光のパワーはバイアス制御回路１１３により制御される。バイアス制御回路１１３は、受光回路５８からの直流電圧信号を参照電圧V_{ref4}と比較してその誤差成分が零になるようにレーザダイオード１１２のバイアス電流を制御する。

【００７０】入力信号光のパワーが比較的ゆっくりと変動したときにバイアス制御回路５９によってレーザダイオード５１からの励起光のパワーが制御される点はこれまでの実施例と同じである。この実施例では、入力信号光のパワーが急激に変化したときに、バイアス制御回路１１３によってレーザダイオード１１２から供給される制御光のパワーが制御される。

【００７１】例えば、入力信号光のパワーが急激に増大した場合に、レーザダイオード１１２から供給される制御光のパワーは増加させられ、これによりドープファイバ５２内における反転分布が急速に減少してこの光増幅器の出力信号光のパワーが一定に保たれる。また、入力信号光のパワーが急激に減少した場合には、レーザダイオード１１２から供給される制御光のパワーが減少させられ、これによりドープファイバ５２内における反転分布が急速に増大して、この光増幅器の信号光出力のパワーが一定に保たれる。

【００７２】制御光の波長はドープファイバ５２で誘導放出を生じさせる波長帯に設定される。例えば、制御光の波長は信号光の波長にほぼ等しく設定される。バイアス制御回路５９における参照電圧V_{ref1}及びバイアス制御回路１１３における参照電圧V_{ref4}は、バイアス制御回路１１３において受光回路５８からの直流電圧信号が参照電圧V_{ref4}に等しいとしたときのこの光増幅器の光出力のパワー（制御光制御目標値）が、バイアス制御回路５９において受光回路５８からの直流電圧信号が参照電圧V_{ref1}に等しいとしたときのこの光増幅器の光出力のパワー（励起光制御目標値）に等しいかそれよりも大きくなるように設定される。即ち、$V_{ref1} \leqq V_{ref4}$である。このような設定を行うようにしているのは、制御光制御目標値が励起光制御目標値よりも小さいと、制御光のパワーの増大を打ち消すように常に励起光のパワーが増加する方向に励起光制御がなされてしまい、良好な制御を行うことができない恐れがあるからである。

【００７３】図１１は図２の本発明の第２構成の他の具体的実施例を示す光増幅器のブロック図である。この実施例が図１０の実施例と異なる点は、光サーキュレータ１１１のポート１１１Ｃから出力される信号光を光分岐回路１２１により分岐し、分岐光の一方に基づき制御光の制御を行っている点である。光分岐回路１２１の他方の分岐光はこの光増幅器の光出力となる。光分岐回路１２１としては光分岐回路５７と同じものが用いられる。

【００７４】受光回路１２２は光分岐回路１２１から供

給された分岐光の平均パワーに応じた直流電圧信号を出力する。バイアス制御回路１２３は、受光回路１２２からの直流電圧信号を参照電圧V_{ref5}と比較してその誤差成分が零になるようにレーザダイオード１１２のバイアス電流を制御する。

【００７５】この実施例によっても、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができる。尚、図１０の実施例におけるのと同じ理由により、バイアス制御回路５９及び１２３における参照電圧V_{ref1}及びV_{ref5}並びに受光回路５８及び１２２における光電変換効率並びに光分岐回路５７及び１２１の分岐比は、バイアス制御回路１２３において受光回路１２２からの直流電圧信号が参照電圧V_{ref5}に等しいとしたときのこの光増幅器の光出力のパワー（制御光制御目標値）が、バイアス制御回路５９において受光回路５８からの直流電圧信号が参照電圧V_{ref1}に等しいとしたときのこの光増幅器の光出力のパワー（励起光制御目標値）に等しいかそれよりも大きくなるように設定される。

【００７６】

【発明の効果】以上説明したように、本発明によると、入力信号光のパワーの急峻な変化にかかわらず出力信号光のパワーを一定に保つことができる光増幅器の提供が可能になるという効果を奏する。

【図面の簡単な説明】

【図１】本発明の光増幅器の第１構成を示すブロック図である。

【図２】本発明の光増幅器の第２構成を示すブロック図である。

【図３】図１の第１構成の第１実施態様を示す光増幅器のブロック図である。

【図４】図１の第１構成の第２実施態様を示す光増幅器のブロック図である。

【図５】図３の第１実施態様の具体的実施例を示す光増幅器のブロック図である。

【図６】光減衰器に入力する信号光のパワー変化の説明図である。

【図７】光減衰器の動作特性の例を示すグラフである。

【図８】図３の第１実施態様の他の具体的実施例を示す光増幅器のブロック図である。

【図９】図４の第２実施態様の具体的実施例を示す光増幅器のブロック図である。

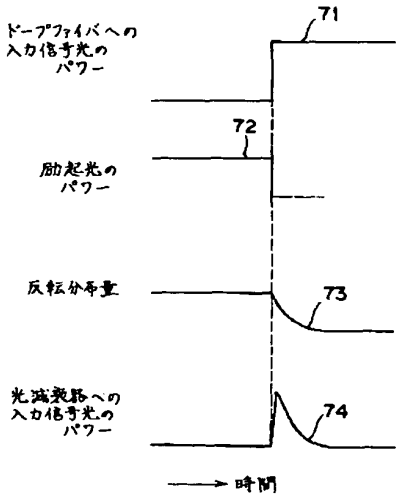
【図１０】図２の第２構成の具体的実施例を示すブロック図である。

【図１１】図２の第２構成の他の具体的実施例を示すブロック図である。

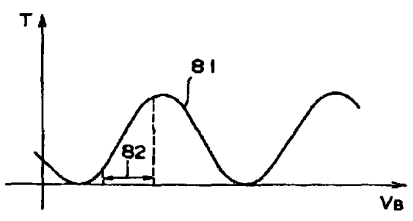
【符号の説明】

１１　励起光源
１２　光増幅媒体
１３　光増幅手段
１４　制御減衰手段
２１，３１，３４　光分岐手段
２２　制御光導入手段
２３　制御光制御手段
３２　励起光制御手段
３３　光減衰器
３５　減衰率フィードバック制御手段
４１　減衰率フィードフォワード制御手段

【図６】

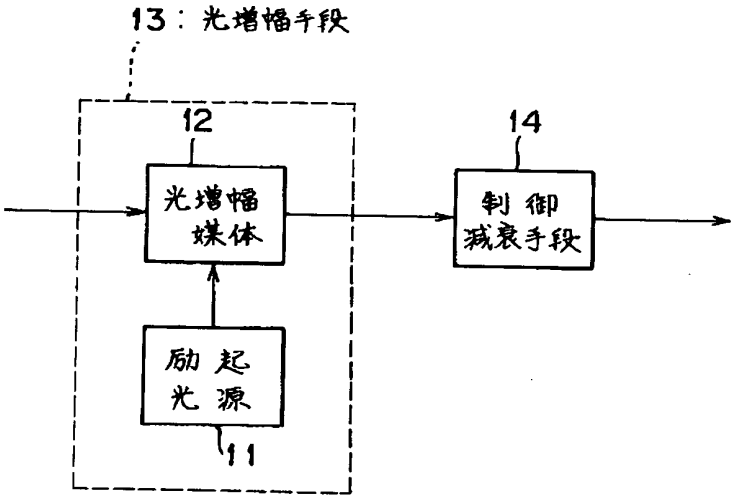


【図７】



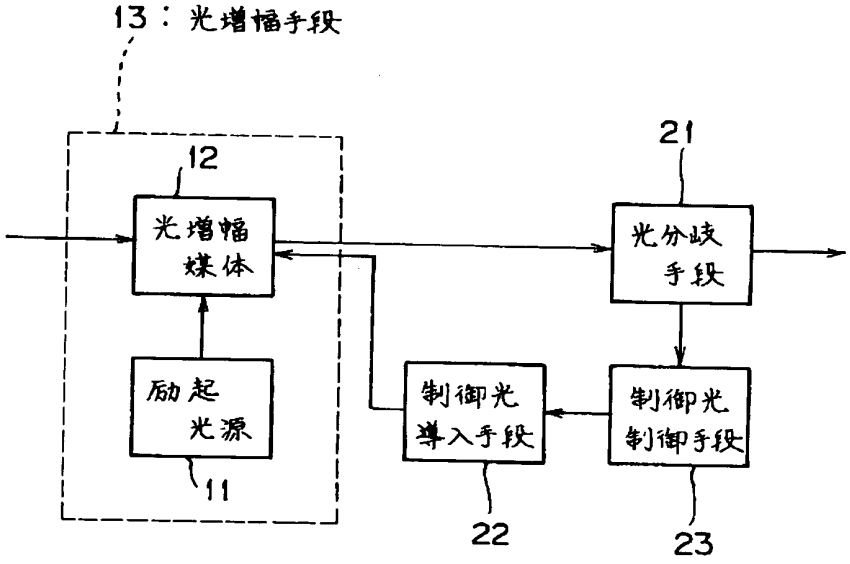
【図1】

第1構成のブロック図



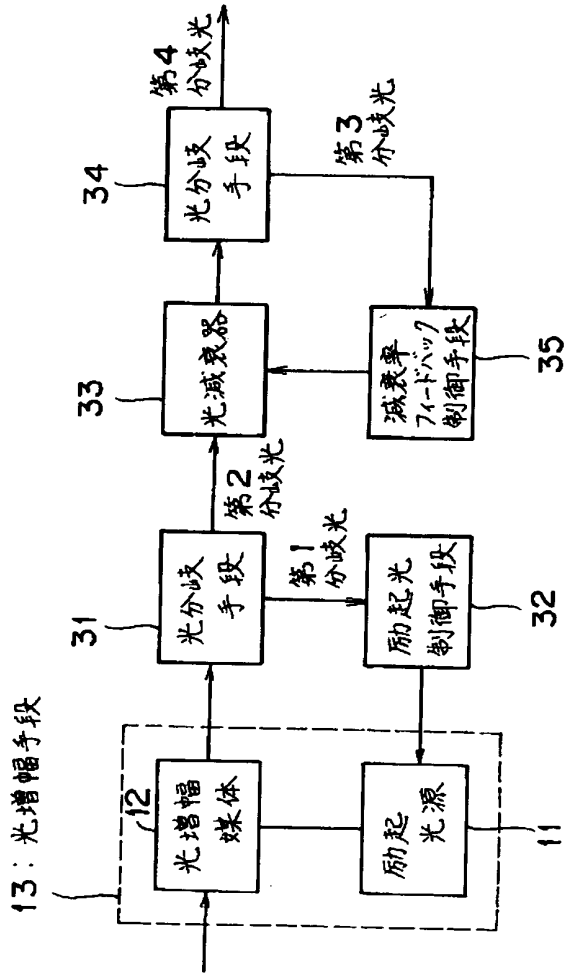
【図2】

第2構成のブロック図



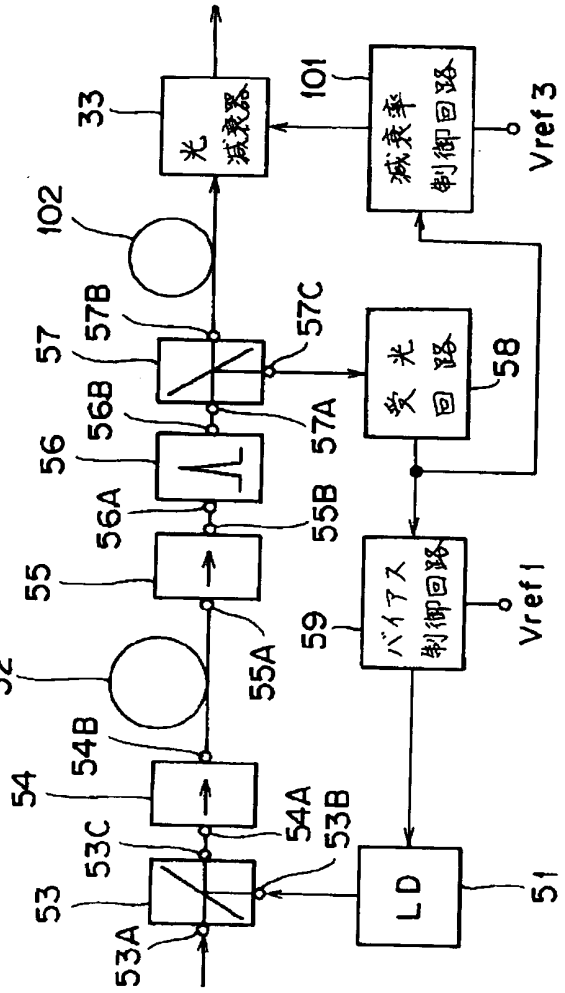
【図3】

第1構成の第1実施態様のブロック図



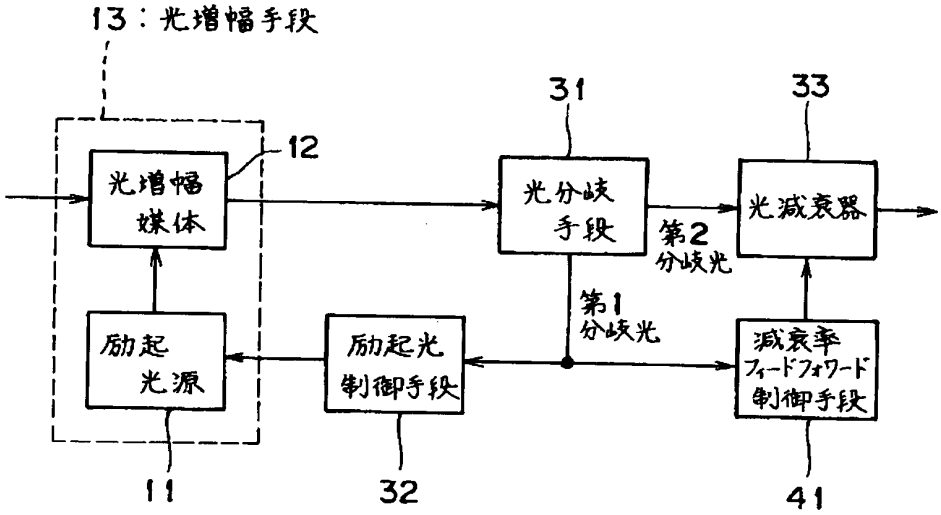
【図9】

図4の具体的実施例を示すブロック図



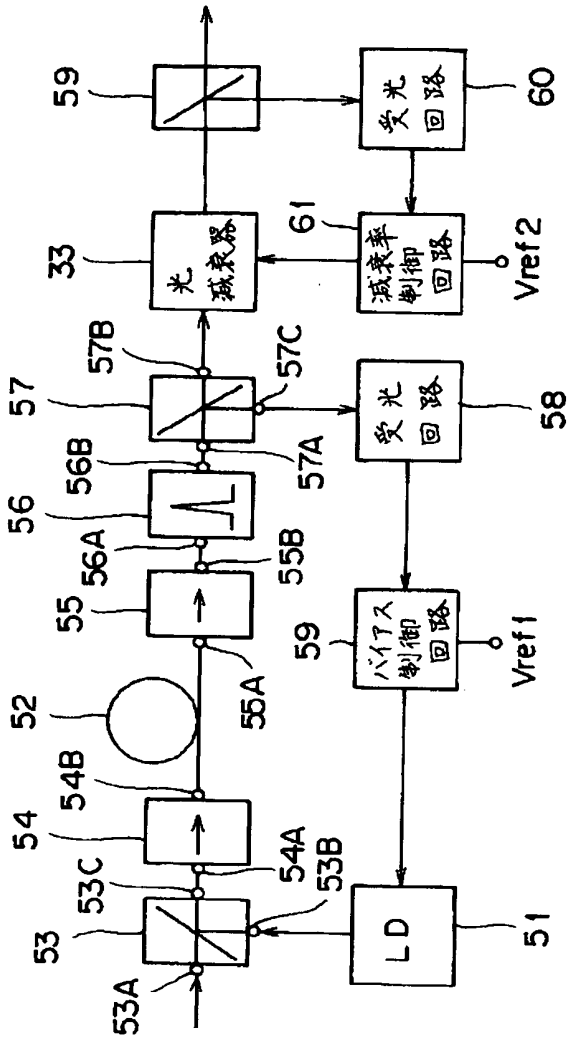
【図4】

第1構成の第2実施態様のブロック図



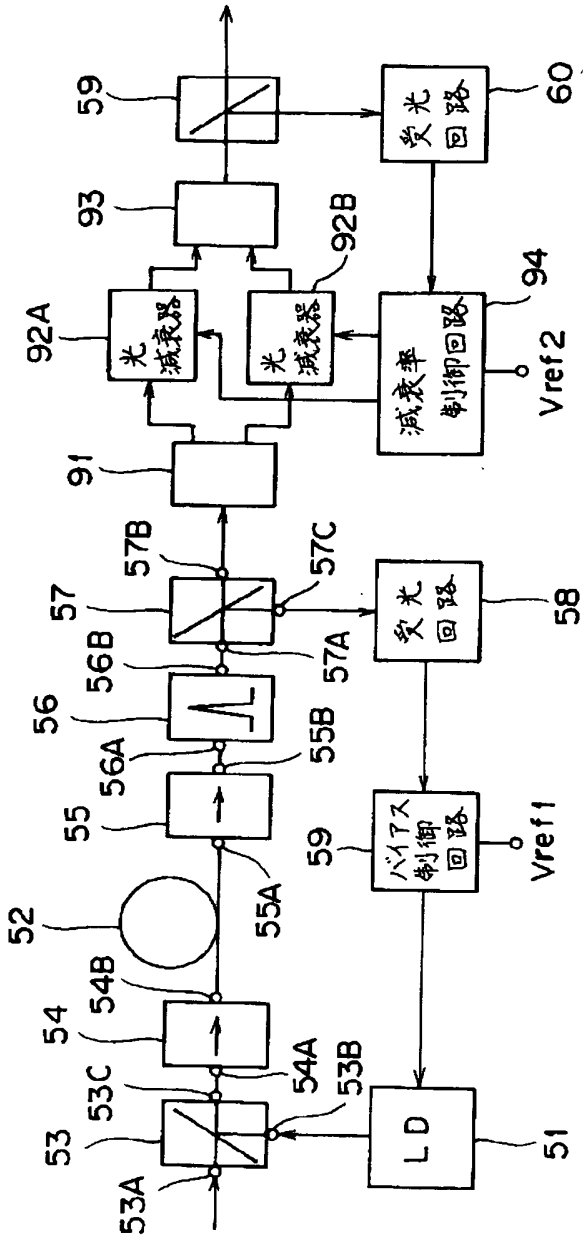
【図5】

図3の具体的実施例を示すブロック図



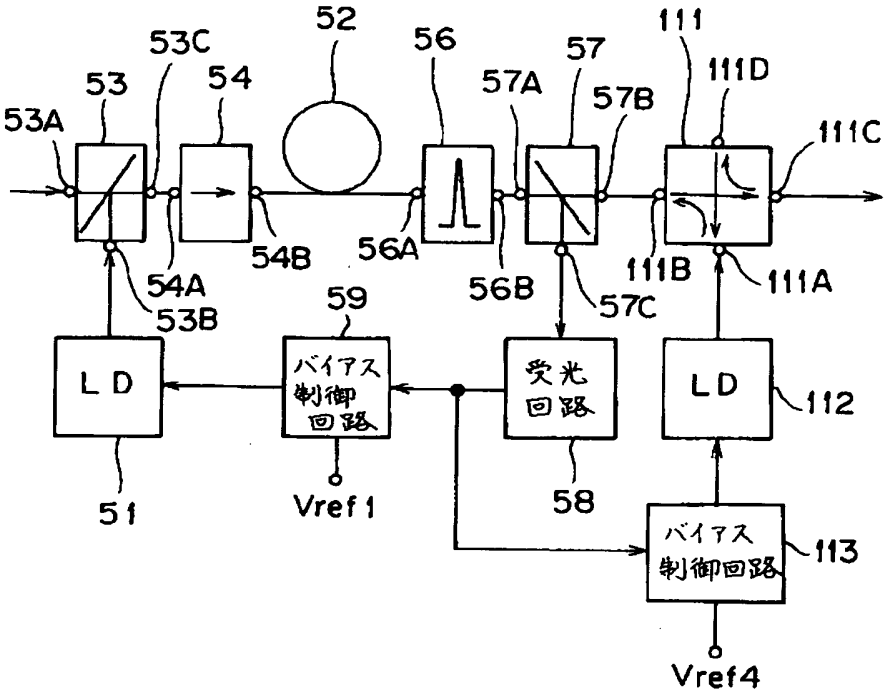
【図8】

図3の他の具体的実施例を示すブロック図



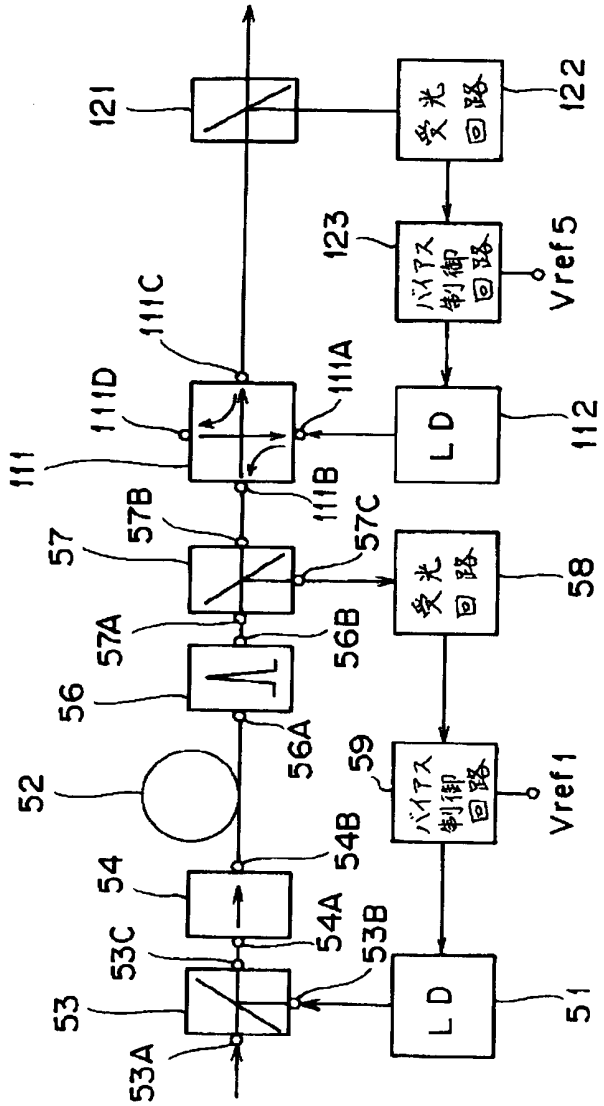
【図１０】

第２構成の具体的実施例を示すブロック図



【図１１】

第２構成の他の具体的実施例を示すブロック図



フロントページの続き

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